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BRICHOS interactions with amyloid proteins and implications for Alzheimer disease

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BRICHOS interactions with amyloid proteins and implications for Alzheimer disease

THESIS FOR DOCTORAL DEGREE (Ph.D.)

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ABSTRACT

To date, about 30 diseases, in which amyloid fibrils form extracellular deposits, have been identified in humans. It is not known if the fibrils have a function, like storage of misfolded proteins, or if they just reflect failure of the cell to manage misfolded proteins. There is no treatment for the majority of the amyloid diseases and therefore disease modifying therapies are sought for. The work in this thesis is focused on studying the BRICHOS domain, which is expressed as part of proproteins found in several protein families involved in a wide range of functions, and some of them are associated with amyloid disease, e.g. interstitial lung disease (proSP-C) and dementia (Bri2). BRICHOS is suggested to have a role in preventing amyloid aggregation of its proproteins. Alzheimer disease (AD) is the most common form of dementia, and aggregation of the amyloid- β peptide ($A\beta$) is widely considered as the causative event. $A\beta$ is derived by sequential cleavages of the $A\beta$ precursor protein $A\beta$ PP. Previous studies have shown that proSP-C BRICHOS reduces $A\beta$ aggregation, and suggested that the monomer is the active form.

In Paper **I** we studied ways to increase the monomer/trimer ratio of proSP-C BRICHOS expressed in *E. coli*, and how this affects its activity against $A\beta$ fibrillation. We found that treatment with amphipathic agents increased proSP-C BRICHOS monomer/trimer ratio and its activity. We also determined that proSP-C BRICHOS is monomeric in mammalian cells.

ProSP-C BRICHOS is only expressed in alveolar type II cells where it facilitates folding of the extremely aggregation prone transmembrane region of proSP-C. In Paper **II** we studied whether proSP-C BRICHOS could reduce amyloid aggregation of a designed amyloidogenic protein in the secretory pathway of mammalian cells. We found that co-expression of BRICHOS led to reduced amyloid aggregation, and prevented subsequent inhibition of proteasomal degradation. This suggests that BRICHOS has generic anti-amyloid properties.

The BRICHOS containing Bri2 and Bri3 proteins are expressed in the central nervous system and have been proposed to be involved in $A\beta$ PP processing. In Paper **III** we studied interactions between Bri2 and Bri3 BRICHOS and endogenous neuronal $A\beta$ PP and $A\beta$. We found that Bri2 BRICHOS is shed from cells, and interacts with intracellular $A\beta$ and $A\beta$ PP. Bri3 BRICHOS was not shed into the extracellular space, showed abundant interactions with intracellular $A\beta$, and exhibited reduced hippocampal and cortical levels in AD.

In Paper **IV** we studied proSP-C and Bri2 BRICHOS effects on $A\beta$ aggregation *in vivo* in a mouse model overexpressing mutant $A\beta$ PP and presenilin1 (PS1). Both proSP-C and Bri2 BRICHOS reduced $A\beta$ levels and aggregation without affecting $A\beta$ PP processing. Mice co-expressing BRICHOS and $A\beta$ PP/PS1 showed improved memory and reduced neuroinflammation compared to $A\beta$ PP/PS1 control animals.

The results in this thesis show that BRICHOS reduces amyloid aggregation *in vitro*, in cells and in a mouse AD model, and indicate a potential physiological relationship between BRICHOS and $A\beta$. These findings together support that BRICHOS and its properties are worth continuing to study in relation to amyloid aggregation and AD.

LIST OF SCIENTIFIC PAPERS

- I. Henrik Biverstål, **Lisa Dolfe**, Erik Hermansson, Axel Leppert, Mara Reifenrath, Bengt Winblad, Jenny Presto, Jan Johansson.
Dissociation of a BRICHOS trimer into monomers leads to increased inhibitory effect on A β 42 fibril formation.
Biochim. Biophys. Acta (2015) 1854, 835–843
- II. **Lisa Dolfe**, Bengt Winblad, Jan Johansson, Jenny Presto.
BRICHOS binds to a designed amyloid forming β -protein and reduces proteasomal inhibition and aggresome formation.
Biochem. J. (2016) 473, 167–178
- III. **Lisa Dolfe**, Simone Tambaro, Helene Bujanova, Marta Del Campo, Jeroen J.M. Hoozemans, Birgitta Wiehager, Caroline Graff, Bengt Winblad, Maria Ankarcrona, Margit Kaldmäe, Charlotte E. Teunissen, Annica Rönnbäck, Jan Johansson, Jenny Presto.
The CNS specific BRICHOS protein Bri3 interacts with neuronal A β 42 and with amyloid plaques in sporadic and familial Alzheimer cases.
Submitted for publication
- IV. Chaeyoung Kim, **Lisa Dolfe**, Krystal C. Belmonte, Luis F. Flores, Aishe Kurti, John D Fryer, Jenny Presto, Jan Johansson, Jungsu Kim.
The molecular chaperone BRICHOS inhibits A β aggregation and other neuropathological phenotypes in a mouse model of A β amyloidosis.
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A molecular chaperone breaks the catalytic cycle that generates toxic A β oligomers.
Nat. Struct. Mol. Biol. (2015) 22, 207-13

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LIST OF ABBREVIATIONS

A β	Amyloid- β
A β PP	Amyloid- β precursor protein
AChEIs	Acetylcholinesterase inhibitors
AD	Alzheimer disease
AICD	A β PP intracellular domain
APLP	A β PP-like protein
APOE	Apolipoprotein E
BACE1	β -site A β PP-cleaving enzyme 1
BBB	Blood brain barrier
Bis-ANS	1,1'-bis (4-Anilino-5,5'-naphthalenesulfonate)
CAA	Cerebral amyloid angiopathy
CNS	Central nervous system
ELISA	Enzyme-linked immunosorbent assay
EM	Electron microscopy
ER	Endoplasmic reticulum
ERAD	ER-associated degradation
FAD	Familial AD
FBD	Familial British dementia
FDD	Familial Danish dementia
FL	Full-length
GFAP	Glial fibrillary acidic protein
GSI	γ -secretase inhibitor
Hsp	Heat-shock protein
IDE	Insulin degrading enzyme
IHC	Immunohistochemistry
ILD	Interstitial lung disease
Immuno-TEM	Immuno-transmission electron microscopy
ITM2B	Integral membrane protein 2B

ITM2C	Integral membrane protein 2C
LOAD	Late-onset AD
LTP	Long-term potentiation
NICD	Notch intracellular domain
NTF	N-terminal fragment
NFTs	Neurofibrillary tangles
PCR	Polymerase chain reaction
PLA	Proximity ligation assay
PM	Plasma membrane
PPCs	Proprotein-like convertases
ProSP-C	Prosurfactant protein-C
PS	Presenilin
<i>PSEN1</i>	Presenilin-1 gene
<i>PSEN2</i>	Presenilin-2 gene
TM	Transmembrane
rAAV	Recombinant adeno-associated virus
SEC	Size exclusion chromatography
SP	Signal peptide
SP-C	Surfactant protein-C
SPPL	Signal peptide peptidase-like
SPR	Surface plasmon resonance
TGN	Trans-Golgi network
UPR	Unfolded protein response
UPS	Ubiquitin proteasome system
WB	Western blot

1 INTRODUCTION

1.1 AMYLOID AND DISEASE

Proteins fold into their native states through a series of steps, in which native interactions are more stable than the alternative ones, which drive the protein to adopt its native structure. Depending on the primary structure and size of the protein, different intermediately folded states are adopted on the way, but the native state is normally the energetically most favorable state. However there is a protein structure that is highly ordered, stable and has even lower energy than the native state, independent of which protein or peptide it is derived from - the amyloid fibril (Jahn and Radford, 2005). The classical definition of amyloid is that it occurs in tissue deposits, stains with Congo red and exhibits green, yellow or orange birefringence when viewed under crossed polars, and is composed of a biochemically characterized protein (Sipe, et al., 2014).

Although amyloid forming proteins and peptides differ in sequences and length, they all form fibrils of β -strands running perpendicular to the fibril axis (Eanes and Glenner, 1968, Landreh, et al., 2016, Nelson, et al., 2005). Many proteins contain segments that can form fibrils under the right conditions *in vitro* (Goldschmidt, et al., 2010) but so far ~30 proteins have been identified, which deposit as extracellular fibrils and cause amyloid disease in humans (Sipe, et al., 2014). Several of these fibrillar deposits are derived from proproteins that after processing give rise to peptides that convert into amyloid fibrils. Examples of such proproteins are the amyloid- β precursor protein (A β PP) and prosurfactant protein-C (proSP-C), involved in Alzheimer disease (AD) and interstitial lung disease (ILD) respectively. Both of these proteins harbor stretches of amino acids that predispose to formation of amyloid fibrils (Kallberg, et al., 2001, Sipe and Cohen, 2000). Amyloid deposits from a specific protein can occur in several tissues and organs, e.g. transthyretin can form amyloid in heart muscle and nervous tissues. Diseases where generalized amyloid occurs are called systemic amyloidosis, whereas localized amyloidosis affects only one organ, e.g. the central nervous system (CNS) in AD.

Data support that it is not a loss-of function but rather gain of toxicity associated with the misfolded and aggregated proteins, which causes several of the amyloid diseases (Stefani and Dobson, 2003, Winklhofer, et al., 2008). It is generally thought that the isolated fibrils studied *in vitro* are structurally the same as the isolated plaque core from amyloid in tissue. Based on this hypothesis, mechanistic studies of amyloid aggregation and potential ways of inhibiting this process are often performed *in vitro*. But there are a number of recurring components associated with amyloid plaques from different diseases, like serum amyloid P component and apolipoprotein E (APOE) that do not form fibrils *in vitro* and it is unclear how these affect amyloid aggregation in a physiological environment (Chiti and Dobson, 2006, Sipe and Cohen, 2000). Some amyloid structures have functional properties, like biofilm produced by bacteria (Bergman, et al., 2016, Fowler, et al., 2007, Sipe, et al., 2014) but it is not known if

the amyloid structures occurring in human disease have a function, like storage of misfolded proteins, or exactly how they convey proteotoxicity (Chiti and Dobson, 2006, Rabinovici and Jagust, 2009, Tanzi and Bertram, 2005), see further below, section 1.5.2. There are proteins that cause intracellular protein inclusions like α -synuclein in Parkinson disease and polyQ repeats of Huntingtin in Huntington's disease, which under certain conditions stain with Congo red and exhibit birefringence. According to the present definition these deposits are not considered as amyloid (Sipe, et al., 2014).

There is no effective treatment for the majority of amyloid diseases, although for some of them extensive efforts have been made to find cures. However, there are some symptomatic treatments available, like acetylcholinesterase inhibitors (AChEIs) for AD (Ankarcrona, 2016). Transthyretin amyloidosis represents an exception where molecules that stabilize the transthyretin tetramer (Hammarstrom, et al., 2003), and thereby reduce dissociation into the amyloidogenic monomer have been developed and approved in clinical trials (Berk, et al., 2013, Coelho, et al., 2013, Coelho, et al., 2012).

1.2 THE COMPLEXITY OF PROTEIN FOLDING AND HOW IT IS MODULATED BY MOLECULAR CHAPERONES

The primary structure of proteins consists of linear chains of amino acid residues, linked together by peptide bonds, and the secondary structures are stabilized by hydrogen bonds forming α -helices or β -sheets. The tertiary structure is built up of from packing of α -helices and β -sheets, as well as unstructured polypeptide segments, by electrostatic and hydrophobic interactions as well as disulfide bonds. Quaternary protein structures are assemblies of multiple folded protein subunits into dimers or larger oligomers of varying complexity.

Small, soluble and relatively stable proteins often efficiently bury their hydrophobic segments in their interior, but they are still at risk to aggregate, especially when they adopt non-native structures under partly denaturing conditions, like e.g. heat shock. Structurally flexible and marginally stable proteins, as well as larger proteins that are slower to fold are especially vulnerable to aggregation in the crowded eukaryotic intracellular environment, where total protein concentrations are as high as 300-400 g/L. Since proteins need to fold into their three-dimensional native structure in order to function, many proteins need the help of molecular chaperones to fold correctly under physiological conditions (Hartl, et al., 2011). Molecular chaperones are proteins that assist in the folding of other proteins but that are not part of the final structures. Moreover they can perform additional functions like preventing interactions between proteins, promote refolding, transportation and degradation of proteins (Hartl, et al., 2011, Muchowski and Wacker, 2005).

Eukaryotic cells maintain their protein homeostasis with the help of a complicated network of chaperones and other factors, collectively referred to as proteostasis. This network influences protein (re)folding, degradation, aggregation, synthesis as well as trafficking inside and outside of cells. Molecular chaperones have a central role in proteostasis, although there are

additional proteins and molecules involved (Balch, et al., 2008). The unfolded protein response (UPR), for example, involves signaling pathways that collectively result in active minimization of aggregation of proteins in the endoplasmic reticulum (ER), by expanding the ER membrane, up-regulating the protein-folding and –degradation machineries, as well as reduction in translation. ER proteins can also be retrogradely transported to the cytosol by the ER associated-degradation pathway (ERAD). In the cytosol ER proteins that have been retro-translocated by ERAD, and cytosolic proteins, can be degraded by the ubiquitin proteasome system (UPS) (Amm, et al., 2014, Walter and Ron, 2011). When proteasome-mediated proteolysis is not efficient enough, ubiquitinated proteins can aggregate into cytoplasmic aggregates called aggresomes, a phenomenon that has been coupled to several protein aggregation diseases (Johnston, et al., 1998, Kopito, 2000). There is likely an age-associated decline of the proteostasis network efficiency, possibly caused by several factors, among them a decrease in proteasome activity, a change in the functional efficiency of molecular chaperones as well as an increase in protein oxidation (Balch, et al., 2008, Landreh, et al., 2016, Muchowski and Wacker, 2005). Neuronal cells are especially vulnerable to aggregated/misfolded proteins because they do not undergo cell division, and thereby lose one possibility to dilute aggregates and renew their intracellular environment. These factors together could be important in explaining the late onset of several neurodegenerative diseases and why age is the main risk factor for AD.

Considering that the majority of proteins harbor segments with potential to form amyloid, but only a fraction of these have been found to cause disease in humans (Goldschmidt, et al., 2010, Sipe, et al., 2014), it seems likely that cells have evolved ways that deal with amyloid aggregation specifically (Balch, et al., 2008, Knight, et al., 2013, Powers and Balch, 2013). Molecular chaperones likely have a role in this defense and several proteins of the heat shock protein (Hsp) family have been shown to accumulate around plaques in AD and with α -synuclein deposits in Parkinson disease (Muchowski and Wacker, 2005). In addition to co-localizing with plaques, cytosolic Hsp70 has been shown to reduce intracellular amyloid- β peptide residues 1-42 (A β 42) mediated proteotoxicity in neuronal cells (Magrane, et al., 2004) and in a *C. elegans* model of A β amyloidosis (Cohen, et al., 2006). The ER resident Hsp70 isoform GRP78 (BiP) has been found to decrease secretion of A β 40/42 and bind to A β PP (Yang, et al., 1998). Extracellular apolipoprotein J (Clusterin) is another chaperone found to co-localize with amyloid plaques in AD (Calero, et al., 2000) and has been shown to reduce A β aggregation and cytotoxicity *in vitro* (Yerbury, et al., 2007). Another protein domain suggested to function as a molecular chaperone and have anti-amyloid activity is the BRICHOS domain (Arosio, et al., 2016, Cohen, et al., 2015, Johansson, et al., 2006, Knight, et al., 2013, Sanchez-Pulido, et al., 2002, Singh and Balch, 2015, Willander, et al., 2011) found in the amyloid disease causing propeptides Bri2 (Sipe, et al., 2014, Vidal, et al., 1999, Vidal, et al., 2000) and proSP-C (Sipe, et al., 2014, H. Willander, et al., 2012a). The BRICHOS domains have been suggested to bind to aggregation prone segments in their respective propeptide (Knight, et al., 2013). Bri2 BRICHOS has been shown to co-localize around

plaques in AD (Del Campo, et al., 2014a). The BRICHOS domain and its anti-amyloid activity are described in more depth in the sections 1.7-1.10 below.

1.3 ALZHEIMER DISEASE

Dementias are the most common type of neurodegenerative diseases. The main form of dementia is AD, accounting for 50-70% of cases. Old age is the primary risk factor for AD, which is becoming an increasing societal and economic problem worldwide due to an ageing population (Prince, et al., 2013). The clinical manifestations of AD often start with the loss of episodic memory, eventually leading to progressive decline in memory and cognitive functions, ultimately causing the afflicted patient to need around the clock care. Patients developing familial dementias, including AD, before age 65, due to mutations, only represent 1-5 % of all dementia cases. The more common late-onset cases presenting after 65 (sporadic dementia) is believed to be multifactorial and at least partly due to cardiovascular risk factors including high cholesterol levels and diabetes, as well as psychosocial risk factors like low education and lack of social and physical activity. However, the relevance of these different factors is debated and old age and carrying one or more *APOE* $\epsilon 4$ alleles are the main established risk factors for AD (Winblad, et al., 2016).

AD was first described in 1906 by Alois Alzheimer, identifying the main histological hallmarks, extracellular neuritic plaques and intracellular neurofibrillary tangles (NFTs) (Reproduced in. (Alzheimer, et al., 1995)). In the 1980's the building blocks of these assemblies, A β and hyper-phosphorylated tau, respectively, were identified (Glennner and Wong, 1984, Iqbal, et al., 1986, Masters, et al., 1985). The neuritic plaques consists mainly of fibrillated A β 42, a 42 residue long peptide originating from A β PP, but other lengths of the A β peptide, e.g. 40 and 43 residues (Welanders, et al., 2009) can also be found. These plaques are intertwined with and surrounded by dystrophic neurons. Other amyloid plaques lacking the dystrophic neurons and a fibrillated compact center, called diffuse plaques, consist almost exclusively of A β peptides ending at position 42 (Gowing, et al., 1994, Iwatsubo, et al., 1994). The diffuse plaques are thought to be preamyloid deposits, acting as precursors to the neuritic plaques (Selkoe, 2001). The NFTs consist of hyper-phosphorylated forms of the microtubule-associated protein tau. The biological function of tau is to promote assembly and maintain the structure of microtubules. Tau is regulated by the degree of phosphorylation, and hyper-phosphorylation causes it to dissociate from the microtubules, eventually leading to impaired axonal transport and synaptic loss (Iqbal and Grundke-Iqbal, 2005, Iqbal, et al., 1986).

Braak staging can be used to separate AD development into different stages, where both amounts of the NFTs and plaques are taken into account, as well as their spread in different regions of the brain. There is variation between patients but generally tau pathology is first observed in the hippocampus and the entorhinal cortex (that transfers information between the hippocampus and neocortex), and eventually affects the neocortex. Amyloid pathology is

initially observed in the basal portions of the neocortex and eventually spreads to most of the neocortex as well as the hippocampus (Braak and Braak, 1991).

The number of NFTs correlates better than plaque load with cognitive decline, but synapse loss shows the strongest correlation with disease severity (Terry, 2000, Terry, et al., 1991). There is a growing body of evidence suggesting that soluble oligomeric A β is neurotoxic and disrupts synaptic plasticity (Kamenetz, et al., 2003, Townsend, et al., 2006, Walsh, et al., 2002) and that these low molecular weight species of A β are responsible for starting the pathogenic cascade that leads to AD (Haass and Selkoe, 2007, Hardy, 2006, Hardy and Higgins, 1992). This could at least partly explain why plaque load does not show a strong correlation with disease progression. Using sensitive techniques like mass spectrometry and enzyme-linked-immunosorbent assays (ELISA), rather than microscopic analysis, it has been shown that there is an increase in soluble A β levels, which correlates with disease severity in AD patients (Lue, et al., 1999, McLean, et al., 1999, Naslund, et al., 2000, Wang, et al., 1999).

In 1984 Glenner and Wong put forward the idea that the gene responsible for production of A β is located on chromosome 21, after isolation and analysis of A β plaques in AD and Downs syndrome (Glenner and Wong, 1984). It was later established that the gene for A β PP is indeed located on chromosome 21 (Rumble, et al., 1989, Tanzi, et al., 1987). Due to trisomy 21, people with Down syndrome have an extra copy of the A β PP gene, and develop AD at a young age. Only ~2% of AD cases are due to autosomal-dominant inherited mutations (Winblad, et al., 2016). Most of these mutations are located in the genes for the A β PP, presenilin-1 (*PSEN1*) or presenilin-2 (*PSEN2*) and almost all of them increase the A β 42 to A β 40 ratio (Tanzi, 2012). The remaining identified mutations in these genes increase A β 42 aggregation or total A β levels (Selkoe, 2001). In addition to the above-mentioned mutations, a rare genetic A β PP variant, which is protective against developing AD, was shown to decrease A β production in cell lines (Jonsson, et al., 2012). These facts all imply that changes in A β levels are important for developing AD.

Sporadic late-onset AD (LOAD) is believed to be a multifactorial disease, combining genetic susceptibility with environmental factors, and there are several risk-associated genes. Some of the identified risk-associated genes for AD are *APOE*, *CRI*, *PICALM*, *CLU* (*APOJ*), *TREM2* and *TOMM40*, implicated in different processes. These include, but are not limited to, lipid metabolism (*APOE*, *CLU*), inflammation (*CRI*, *TREM2*), A β aggregation (*CLU*) and endosome recycling (*PICALM*) (Tanzi, 2012, Winblad, et al., 2016). *APOE* remains the only well established risk factor and three different alleles exist - ϵ 2, ϵ 3 and ϵ 4 - where ϵ 4 increases the risk of developing AD fourfold when inherited in one copy and tenfold when inherited in 2 copies (Tanzi, 2012). *APOE* is involved in cholesterol metabolism (Bjorkhem and Meaney, 2004) and is thought to be involved also in A β clearance in AD (Tanzi, 2012). These risk factors reflect some but not all of the processes involved in AD, like inflammation, oxidative stress, cytoskeletal degeneration, and lipid metabolism, which indicate that regardless of what causes AD, once initiated, several biological processes are affected.

Other than the “A β cascade hypothesis” that postulates that A β accumulation and aggregation causes the pathogenic cascade eventually leading to synaptic alterations, inflammation, tau hyper-phosphorylation, neuronal loss and cognitive decline (see section 1.5) there are a number of other possible explanations for the cause of AD. One hypothesis is that the cytoskeletal degeneration and formation of NFTs cause AD (Braak and Braak, 1991), which is supported by the correlation between NFTs and cognitive decline. However mutations in tau do not cause AD but instead frontotemporal dementia (Goedert and Jakes, 2005). NFTs are found in several other neurodegenerative diseases with no A β deposition, perhaps suggesting that in AD, the tangles arise as a secondary effect to gradual accumulation of A β . Another hypothesis is that inflammation causes AD. Activated microglia and reactive astrocytes accompany the NFTs and neuritic plaques (Akiyama, et al., 2000) and inflammation could initially have a protective role by clearing A β via phagocytosis (Glass, et al., 2010). It has been shown that A β can activate microglia, leading to the production of reactive oxygen species, which will cause neuronal dysfunction and eventually cell-death (Akiyama, et al., 2000, Glass, et al., 2010). Whether these processes are a cause or an effect of the disease is, however, not known. The “cholinergic hypothesis” is based on the observed loss of cholinergic neurons in AD (Bartus, et al., 1982), and led to the development of AChEIs as symptomatic treatment. AChEIs, however, only have moderate effects on improving memory and no effects on neurodegeneration, and therefore disease-modifying treatments of AD are intensely sought for.

1.4 AMYLOID PRECURSOR PROTEIN (A β PP) AND AMYLOID- β PEPTIDE (A β)

A β PP is a single-pass type I transmembrane (TM) protein, with its N-terminus in the ER lumen or the extracellular space. There are several splice variants of mammalian A β PP, and A β PP695 (i.e. containing 695 residues) is the main CNS isoform and is referred to as A β PP in this thesis, unless otherwise stated. Other forms (A β PP770 and A β PP751) are expressed in other tissues as well as in the CNS, and several additional variants are expressed in various parts of the body. Human A β PP is part of a family, here referred to as the APP family including the A β PP-like protein 1 and 2 (APLP1 and APLP2) (van der Kant and Goldstein, 2015). This family is well conserved, found in vertebrates and in some invertebrates, but not in prokaryotes, plants or yeast, suggesting that the occurrence of the APP family coincides with the evolution of the nervous system (Shariati and De Strooper, 2013). A β PP, including its different isoforms is the only family member containing an A β peptide domain. The intracellular C-terminal of A β PP is short and unstructured, while the extracellular N-terminal domain correspond to the largest part of the protein. The C-terminal part is conserved in the A β PP, APLP1 and APLP2 (van der Kant and Goldstein, 2015) and experimental data suggest that the hydrophobic C-terminal region interacts with the lipid bilayer (Barrett, et al., 2012).

There is more divergence in the N-terminal part between both APP-family members and A β PP splice variants.

To deduce the function of the APP family, loss of function studies have been the standard, and experiments knocking out the homologues in *C. elegans* and *D. melanogaster* suggest a role in axonal outgrowth and synapse formation (van der Kant and Goldstein, 2015). Studies focusing on the mammalian APP family have given a more complex picture. Single knockouts of A β PP, APLP1 and APLP2 in mice are viable (Shariati and De Strooper, 2013, van der Kant and Goldstein, 2015). Although A β PP knockouts are viable they have reduced body weight, reduced locomotor activity (Zheng, et al., 1995), axonal transport defects (Goldstein, 2012, Smith, et al., 2007, Smith, et al., 2010) and several additional phenotypic traits. Double knockouts for A β PP and APLP2 or APLP1 and APLP2 die after birth whilst double knockouts for A β PP and APLP1 are viable (Heber, et al., 2000). This suggests that APLP2 can compensate for the loss of both A β PP and APLP1 but if this reflects functional redundancy or not is debated (Shariati and De Strooper, 2013). The studies of A β PP function point towards a role in intracellular signaling implicated in axonal and dendritic processes as well as supporting synaptic maintenance (van der Kant and Goldstein, 2015). Whether the unprocessed full length A β PP and/or its processing products perform these functions is not known.

1.4.1 A β PP processing

A β PP undergoes posttranslational modifications and sequential cleavages by α -, β - and γ -secretases. Neuronal A β PP is found in both postsynaptic and presynaptic compartments (DeBoer, et al., 2014), and it has been suggested that its processing can be regulated by synaptic activity (Kamenetz, et al., 2003). There are two main A β PP processing pathways, often referred to as the “amyloidogenic” and the “non-amyloidogenic” pathway. The first cleavage in the non-amyloidogenic pathway is within the A β sequence and is performed by the α -secretase. This generates a soluble A β PP ectodomain referred to as sA β PP α and a membrane bound C-terminal fragment, called C83 or CTF α . The C83 fragment is either degraded, or cleaved by the γ -secretase complex within the TM region into short peptides, collectively called p3, including A β 17-40 and A β 17-42. Additionally the A β PP intracellular domain (AICD) is released by the γ -secretase cleavage. The α -secretase is not one specific secretase, but a family of proteases called ADAM and especially ADAM9, ADAM10 and ADAM17 are responsible for cleavage of A β PP. The γ -secretase is a multi-subunit aspartyl protease consisting of the presenilin 1 and/or 2 (PS1, PS2) making up the catalytic core and accessory proteins nicastrin, anterior pharynx-defective 1 and PS enhancer protein 2 (De Strooper, et al., 2010, Nhan, et al., 2015). The name non-amyloidogenic refers to the fact that α -secretase cleaves within the A β sequence of A β PP, preventing the generation of A β . However, the p3 fragments are released and have been found in diffuse plaques (Gowing, et

al., 1994). The ADAM proteases are located at the plasma membrane (PM) (Sisodia, 1992). The location of the γ -secretase complex is debated, but data points towards that γ -secretase cleavage takes place either in the late endosome/lysosome compartment (Takahashi, et al., 2002, Vieira, et al., 2010), or in the trans-Golgi network (TGN) (Choy, et al., 2012). In the amyloidogenic pathway, instead of α -secretase, β -secretase cleaves A β PP generating the soluble ectodomain sA β PP β and a membrane bound C-terminal fragment, called C99 or CTF β (De Strooper, et al., 2010, Nhan, et al., 2015). The main β -secretase is the β -site A β PP-cleaving enzyme 1 (BACE1). BACE1 requires low pH for optimal performance, and its activity is most likely localized to acidic intracellular compartments like endosomes and the TGN (De Strooper, et al., 2010). After BACE1 cleavage, γ -secretase cleaves C99 in the TM domain generating A β peptides of different length, ending at residue 38-43 (LaFerla, et al., 2007). Gamma secretase cleavage also releases an identical AICD domain as generated in the non-amyloidogenic pathway (van der Kant and Goldstein, 2015). See Figure 1 for an overview of the two main A β PP processing pathways.

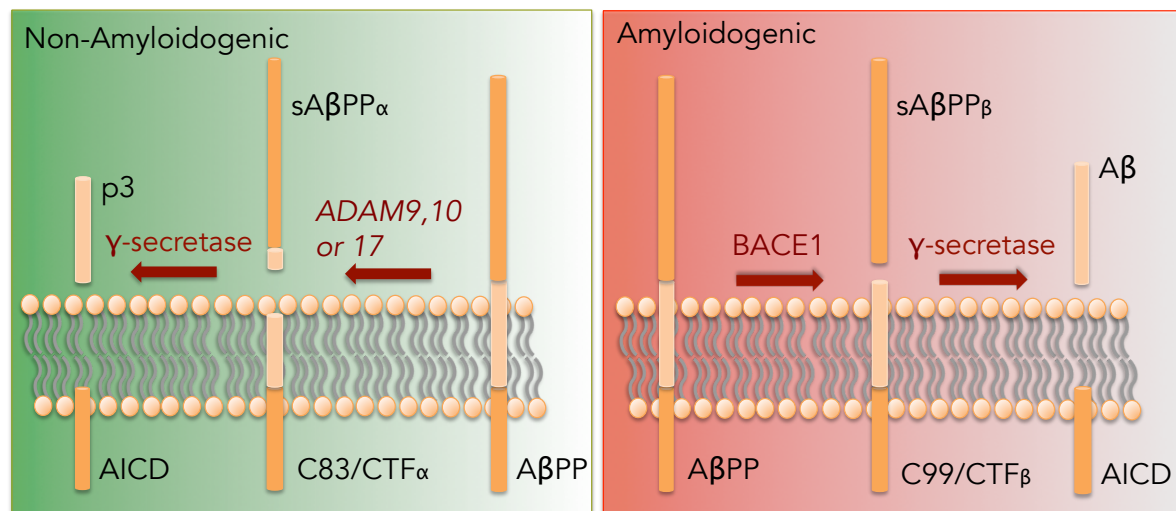


Figure 1. A β PP processing. ADAM9, 10 or 17 cleaves A β PP in the non-amyloidogenic pathway, generating sA β PP α and C83 fragments. γ -secretase then cleaves C83 by intramembrane-proteolysis into AICD and p3. BACE1 cleaves A β PP in the amyloidogenic pathway, generating sA β PP β and C99, and C99 is subsequently cleaved by γ -secretase into AICD and A β . Adapted from LaFerla et al, 2007 (LaFerla, et al., 2007).

1.4.2 A β

Several of the A β PP processing products have been shown to possess neurotoxic and/or neuroprotective effects, but A β is by far the most studied (Nhan, et al., 2015, van der Kant and Goldstein, 2015). When discovered, A β was thought to be solely pathogenic, but it was later realized that A β is continuously produced in healthy brains and released during neuronal activity (Puzzo and Arancio, 2013). A variety of functions have been suggested for A β , from being a signaling molecule, a transcriptional factor, a cholesterol transport regulator and anti-microbial agent (Nhan, et al., 2015, Puzzo and Arancio, 2013). Furthermore, it has been suggested that physiological concentrations of A β are necessary for normal synaptic plasticity

and memory function (Puzzo and Arancio, 2013), perhaps by modulating vesicle cycling (Abramov, et al., 2009), or by inhibiting as well as facilitating presynaptic release of excitatory neurotransmitters (Mura, et al., 2012). So far no unequivocal biological function of A β has been established.

A β 40 represents about 90% of all secreted A β fragments, while the more aggregation prone A β 42 and A β 43 constitute only a small fraction. However, the longer fragments, A β 42 and A β 43 are the predominant species found in AD plaques (Sisodia and St George-Hyslop, 2002). An interesting observation is that the main part of intraneuronal A β is A β 42 and not A β 40 (Gouras, et al., 2000). It is not known if this A β 42 is internalized from the extracellular pool, or generated intracellularly. Data suggest that the main part of A β is produced in the endosomal pathway and a smaller fraction is generated in the ER and Golgi (LaFerla, et al., 2007, Selkoe, 2001, Sisodia and St George-Hyslop, 2002). A β can be degraded by several pathways but the main degradation pathways *in vivo* are by neprilysin and insulin degrading enzyme (IDE) (Farris, et al., 2003, Iwata, et al., 2000, Qiu, et al., 1998, Yasojima, et al., 2001). Regardless of its biological function, if any, A β in its oligomeric/fibrillar forms has proteotoxic properties, and is deposited in plaques in AD brain. A deeper view on proteotoxicity is given in section 1.5.2.

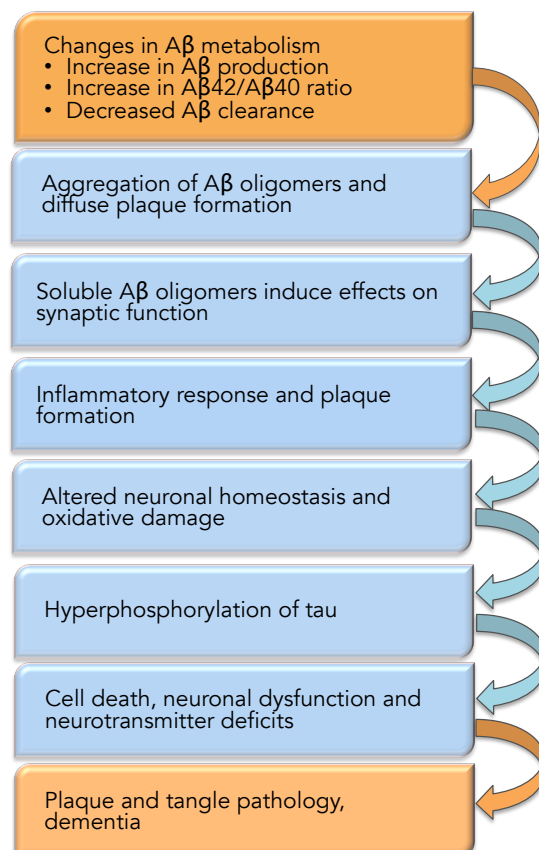


Figure 2. Summary of the amyloid cascade hypothesis. Adapted from Haass et al, 2007 (Haass and Selkoe, 2007).

1.5 THE AMYLOID CASCADE HYPOTHESIS

The amyloid cascade hypothesis postulates that aggregation of A β starts the pathogenesis leading to AD, while synaptic alterations, inflammation, tau hyper-phosphorylation, neuronal loss and cognitive decline are downstream events. A β as such, without subsequent aggregation is not sufficient to cause AD since it is present throughout life in the CNS (Seubert, et al., 1992, Walsh, et al., 2000). This hypothesis is still debated but a number of factors support its validity (Hardy and Selkoe, 2002, Hardy and Higgins, 1992). After the isolation of the A β peptide in the 1980's (Glenner and Wong, 1984, Masters, et al., 1985) and the subsequent identification of the A β PP gene on chromosome 21 (Tanzi, et al., 1987), early-onset familial AD (FAD) mutations were identified. To date, hundreds of

mutations in the A β PP, PSEN1 and 2 genes have been identified. These mutations all

increase A β production, increase the A β 42/A β 40 ratio or increase A β aggregation propensity (Karran and De Strooper, 2016). The A β PP mutations only account for a fraction of all FAD mutations and the majority is located in the PS's (Tanzi and Bertram, 2005). The PS mutations supposedly cause AD because of an increase in A β 42 production (Scheuner, et al., 1996). It has been argued that mutations only account for 1-5% of AD cases and that FAD and LOAD could represent different diseases. However, the pathological changes are the same, the only difference is the age of onset, suggesting that they likely represent one and the same disease (Karran and De Strooper, 2016, Selkoe, 2001). You could argue that this support that the familial mutations and their effects are relevant to the understanding of the pathogenesis of also the sporadic AD. AD patients have elevated cerebral A β levels, caused by increased production in the familial cases, and it is possible that decreased A β clearance and degradation are more important factors in LOAD (Tanzi and Bertram, 2005). The second main risk factor for AD is ApoE status. There is evidence that ApoE is involved in deposition of amyloid (Bales, et al., 1999, Holtzman, et al., 1999, Jack, et al., 2015, Schmechel, et al., 1993) and *APOE* ϵ 4 allele carriers have higher steady state levels of A β in the brain (Selkoe, 2001). Individuals with Down's syndrome develop AD at a young age, likely due to overproduction of A β PP and its downstream product, A β (Tanzi, 2012). The only so far identified protective mutation against AD (Jonsson, et al., 2012) reduces A β production (Benilova, et al., 2014, Maloney, et al., 2014). Taken together, these observations indicate that A β is the underlying cause of the disease. The original amyloid cascade hypothesis coined in the 90's (Hardy and Higgins, 1992) have now been revised to more focus on soluble oligomeric species of A β as the toxic moiety (Haass and Selkoe, 2007, Karran and De Strooper, 2016, Tanzi and Bertram, 2005). The amyloid cascade hypothesis is summarized in Figure 2.

One of the arguments against A β aggregation as the initiator of AD is that elderly non-demented individuals can have substantial amount of diffuse plaques in their brains (Dickson, 1997). However soluble levels of A β , including oligomers show better correlation with cognition than plaque count (Lue, et al., 1999, McLean, et al., 1999, Naslund, et al., 2000, Wang, et al., 1999). An argument supporting that soluble A β , presumably oligomers, are toxic is that A β PP transgenic mice show memory deficits and changes in neuron function already before amyloid deposition (Chapman, et al., 1999, Dineley, et al., 2002, Wu, et al., 2004). Moreover synthetic A β and soluble oligomers of secreted A β can inhibit hippocampal long-term potentiation (LTP) (Kamenetz, et al., 2003, Lambert, et al., 1998, Townsend, et al., 2006, Walsh, et al., 2002), an electrophysiological mechanism underlying learning and memory. Cognitive function was impaired after injecting conditioned medium from A β producing cells in rats. This effect could be abolished by immunodepletion of soluble, but not monomeric A β , suggesting that the effects were caused by oligomeric A β (Cleary, et al., 2005). However so far it is not certain if the large insoluble deposits or the small soluble oligomers represent the dominant toxic moiety and more research is needed to settle this question (Haass and Selkoe, 2007). It is not even known if it is intercellular or extracellular

A β , or both, that are involved in the pathogenesis of AD. Much focus has been on extracellular A β since plaques are extracellular, but there are studies suggesting that intraneuronal A β accumulation could be an early process in AD (Hartmann, 1999, LaFerla, et al., 2007). Intracellular accumulation precedes extracellular plaque formation in individuals with Down's syndrome (Gyure, et al., 2001). Moreover experimental data support the existence of intraneuronal oligomerization of A β (Walsh, et al., 2000), highlighting a possible link between intracellular and extracellular aggregation.

One of the main criticisms against the amyloid hypothesis today, is that several clinical trials, testing amyloidcentric drugs have failed to meet their endpoints (Karran and De Strooper, 2016). Support for the anti-amyloid strategy, however, comes from trials of antibodies raised against A β . One study targeting monomeric A β with a monoclonal antibody was hypothesized to lead to lowering of A β steady state levels and thereby reduced plaque load, and the trial showed positive effects on cognition in mild AD groups (Siemers, et al., 2016). A second antibody targeting A β aggregates was recently shown to reduce plaque load and a slowing of clinical decline in prodromal to mild AD groups (Sevigny, et al., 2016). Further clinical trials of this and other amyloidcentric drugs are being conducted. Some of these will hopefully be successful and/or give better insight to whether targeting A β is a viable way of treating AD (Karran and De Strooper, 2016). The clinical trials have highlighted that more studies, both *in vitro* and *in vivo* are needed to understand A β aggregation mechanisms, in particular ways to specifically target the toxic species. For example, there are concerns that if the soluble oligomers are the toxic species, then targeting the plaques could actually release more oligomers and rather increase the toxicity.

1.5.1 Mechanisms of A β aggregation

The mechanisms of A β aggregation can be divided into several steps. In the primary nucleation event A β monomers interact and form oligomers, and the concentration of A β monomers is rate determining in this step. Secondary events are dependent on the concentration of existing fibrils, and can be divided into monomer-independent and monomer-dependent steps. The monomer-independent step is fragmentation, which only depends on the fibril concentration. The monomer-dependent steps are the secondary nucleation event and elongation. These steps are dependent on both monomer and fibril concentrations, see Figure 3. In the secondary nucleation step, the surfaces of already existing fibrils catalyze the generation of oligomers from monomers. (Cohen, et al., 2013, Ruschak and Miranker, 2007). Data point towards the secondary nucleation event being the major pathway responsible for the generation of toxic A β oligomers (Arosio, et al., 2016, Cohen, et al., 2015).

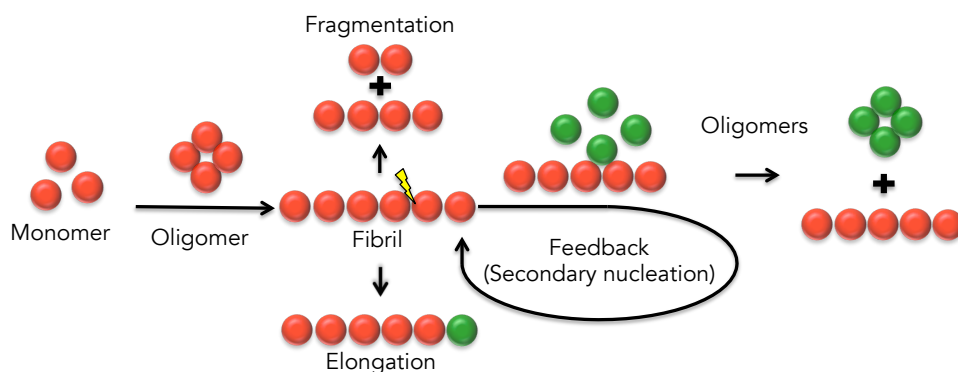


Figure 3. Aβ aggregation mechanisms. Monomers form oligomers in a primary nucleation event, eventually forming fibrils. **Fragmentation**; Fibrils fragment and new fibrils are formed in a monomer-independent step. **Elongation**; Fibrils are elongated by the addition of monomers, in a monomer-dependent step. **Secondary nucleation**; Oligomers form from monomers at the surface of existing fibrils, in a monomer-dependent step. Adapted from Cohen et al, 2013 (Cohen, et al., 2013).

1.5.2 Proteotoxicity

How proteotoxicity in general is conveyed is not clear although there are several ideas. The toxicity of the oligomers is thought to correlate with their exposure of hydrophobic surfaces and polypeptide backbone moieties, which have not been incorporated in the fibrillar core (Bolognesi, et al., 2010). In a study using soluble oligomers from several amyloidogenic peptides including Aβ, it was shown that oligomer toxicity from different peptides was inhibited by co-incubation with a monoclonal antibody raised against oligomers of Aβ. It was proposed that oligomer toxicity is conveyed through a common oligomeric conformational epitope that is not sequence dependent (Kayed, et al., 2003). The “amyloid pore” hypothesis proposes that oligomers form ring-like structures, exposing hydrophobic regions and cause toxicity by forming pores in membranes, and there is experimental data supporting that Aβ can permeabilize membranes (Caughey and Lansbury, 2003, Volles and Lansbury, 2003). Another hypothesis is that misfolded proteins, together with a decline in protein quality control, lead to disruption of cellular homeostasis and neurodegeneration (Balch, et al., 2008). By expressing designed amyloid forming proteins in cells and analyzing the interactome by quantitative proteomics, it was found that cytosolic aggregates of these proteins sequester large multifunctional proteins, possibly causing cytotoxicity (Olzscha, et al., 2011). This study support that amyloid aggregation disrupts cellular homeostasis, but if the above mechanisms are relevant to AD pathogenesis is not certain. An additional complexity in understanding protein aggregation and its effects is that there are variations depending on the subcellular location of the proteins. For example, expressing polyQ peptide repeats of pathological length in the ER, results in more soluble protein and less aggregation than when the same proteins are expressed in the cytosol (Rousseau, et al., 2004).

1.6 TREATMENT OF AD

There is only symptomatic treatment available for AD today and the interest for disease-modifying therapies is high. The current treatments are AChEIs and one NMDA (N-methyl-D-aspartic acid) receptor antagonist. There is a reduction in the synthesis of acetylcholine in AD and AChEIs are used to prolong the effects of acetylcholine in the synaptic cleft (Kulshreshtha and Piplani, 2016). AChEI treatment is approved for mild to moderate AD but only has limited effects on cognition and no effect on neurodegeneration. The NMDA receptor antagonist, Memantine is approved for moderate to severe AD and prevents glutamate-mediated neurotoxicity (Danysz, et al., 2000). The extensive failures of conducted clinical trials have generated an interest to develop sensitive biomarkers for AD, in order to enable earlier and more secure diagnosis as well as monitoring disease progression during treatment. Studies suggest that AD pathology begins decades before clinical symptoms arise (Bateman, et al., 2012). Validated biomarkers would facilitate more effective monitoring and evaluation of clinical trials. Another effect from unsuccessful clinical trials is that patients with early-stage AD are now included, and imaging techniques are used to verify the presence of A β plaques as inclusion criteria.

Most of the drugs in clinical trials for AD focus on reducing A β concentration in the brain or increase A β clearance. The strategies include inhibiting the secretases cleaving A β PP, thereby inhibiting the production of A β , as well as passive or active immunotherapies to increase A β clearance from the brain. Several γ -secretase inhibitors (GSI) have been tested, for example Semagacestat, but unfortunately these trials have been terminated due to unacceptable side effects (De Strooper and Chavez Gutierrez, 2015). All of the secretases involved in A β PP cleavage have numerous substrates, and for example Notch1 is a substrate of γ -secretase. Gamma secretase cleavage of Notch1 releases the Notch intracellular domain (NICD) involved in signaling pathways in neurogenesis and embryonic development (De Strooper, et al., 1999). Notch function is thus obviously important and it is believed that several of the side effects caused by GSI's occur because of inhibition of Notch processing. When Semagacestat was tested in a phase III clinical trial it led to side effects such as weight loss, skin cancer and infections, but even more importantly it led to faster decline in cognition, the opposite of the intended outcome (De Strooper and Chavez Gutierrez, 2015, Doody, et al., 2013). This failure likely contributes to the fact that only one GSI is in clinical trial now. Another option is BACE1 inhibitors meant to decrease β -secretase cleavage of A β PP and thus the generation of C99 and eventually A β . Several such inhibitors are in phase III trials (Ankarcrona, 2016). BACE1 have ~20 identified substrates and it is not clear what chronic inhibition of BACE1 will lead to (De Strooper and Chavez Gutierrez, 2015). Another concern with inhibiting A β production through modulating A β PP cleavage is that it would also effect the other processing products of A β PP, like AICD that similarly to NICD is implicated in important signaling pathways (Pardossi-Piquard and Checler, 2012). Passive and active immunotherapies intend to enhance clearance of A β from the brain and are currently in clinical trials. The first vaccine tested against AD caused encephalities and

patients showed no clinical improvement as regards AD symptoms (Gilman, et al., 2005,Orgogozo, et al., 2003). Another vaccine, CAD106, showed positive results in phase II trials are now going into phase III trials. Passive immunotherapies in clinical trials include the antibodies Solanezumab now in an additional phase III trial, and Gantenerumab designed to bind A β fibrils (Ankarcrona, 2016) as well as Aducanumab designed to bind A β aggregates (Sevigny, et al., 2016). The monoclonal antibody Bapineuzumab, targeting N-terminal A β with the aim of binding to plaques and increase microglial activation, recently failed in clinical trials (Karran and De Strooper, 2016,Karran and Hardy, 2014). Additional treatment targets for AD in ongoing clinical trials include tau phosphorylation, inflammation, cholesterol therapeutics and several more, see Figure 4.

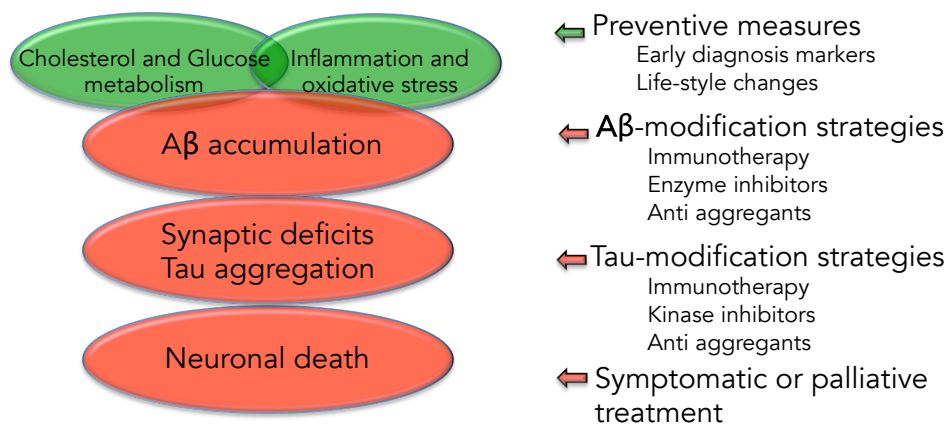


Figure 4. Overview over AD treatment strategies. Adapted from Winblad et al, 2016 (Winblad, et al., 2016).

As mentioned earlier, there are those who suggest that the many failures of amyloidocentric drugs disprove the amyloid cascade hypothesis. However, also drugs aimed at reducing tau pathology (Medina and Avila, 2014,Morimoto, et al., 2013) and other targets, such as cholinesterase inhibitors have failed in clinical trials (Schneider, et al., 2014). It is likely that the problems with efficacy and positive outcomes are at least partially due to the multifactorial nature of AD, problems with crossing the blood brain barrier (BBB) as well as incomplete preclinical data. The animal models of AD used for preclinical testing are not fully mimicking the human disease, and therefore, results from drug testing in these models are not reliable for predicting the outcome in patients. These factors together with the lack of good biomarkers and well-defined target populations can probably explain why so many clinical trials have failed (Winblad, et al., 2016).

Targeting proteostasis to promote anti-aggregation, by up-regulation of chaperones or degradation machineries like the proteasome could be another strategy for treating AD. So far chaperone-targeting treatments have only been tested in preclinical settings (Ankarcrona, 2016). An interesting feature of such a strategy is that it could possibly be applied to more than one neurodegenerative protein aggregation disease. Discouraging, however, is that similar to inhibiting enzymes it could lead to a number of side effects due to modulation of molecular machineries involved in several off-target processes. For proteostasis modulation

to be a possible treatment strategy it is thus important to achieve enough specificity so that unacceptable side effects are avoided.

1.7 THE BRICHOS DOMAIN

The BRICHOS domain was first described in 2002 and consists of ~100 amino acid residues (Sanchez-Pulido, et al., 2002). BRICHOS has been identified in about 10 human protein families and the name is derived from the proteins Bri2, Chondromodulin-I and surfactant protein C (SP-C). The proteins containing a BRICHOS domain have a wide range of functions and disease associations including ILD (proSP-C), dementia (Bri2) as well as cancer (Chondromodulin-I and Gastrokines) (Sanchez-Pulido, et al., 2002, H. Willander, et al., 2012a). The work in this thesis is focused on the BRICHOS domains from proSP-C, Bri2 and Bri3. There are low pairwise sequence identities between the different BRICHOS domains (~15-25%) but they have similar predicted secondary structures. Their precursor proteins all have a common overall architecture, and are predicted to be type II TM proteins (Hedlund, et al., 2009, Knight, et al., 2013, Sanchez-Pulido, et al., 2002), i.e. the N-terminal is located in the cytosol. Integral membrane protein 2B (ITM2B) also called Bri2 and Bri3 (ITM2C) are part of the BRI family. Bri2 and Bri3 share 42% overall sequence identity, and comparing the BRICHOS domains of Bri2 and Bri3 show that they have 60% sequence identity, indicating that they perform similar functions. The BRI family could be the oldest family of BRICHOS containing proteins, considering it has members from the most ancient species (flies and worms) (Sanchez-Pulido, et al., 2002).

All BRICHOS containing proproteins have an N-terminal cytosolic part, a hydrophobic TM region, a linker region followed by a BRICHOS domain, and a C-terminal region. The only exception is proSP-C, which has no additional C-terminal region following the BRICHOS domain. All of these proproteins have a segment with high β -sheet propensity, i.e. the C-terminal region, except in proSP-C, where instead the TM region has high β -sheet propensity (Hedlund, et al., 2009, Sanchez-Pulido, et al., 2002). There are three strictly conserved residues in all BRICHOS domains, two cysteines and one aspartic acid. The two cysteines form a disulfide bridge in proSP-C BRICHOS, and their strict conservation suggest that a corresponding disulfide bridge is present in all BRICHOS domains (Willander, et al., 2011). See Figure 5.

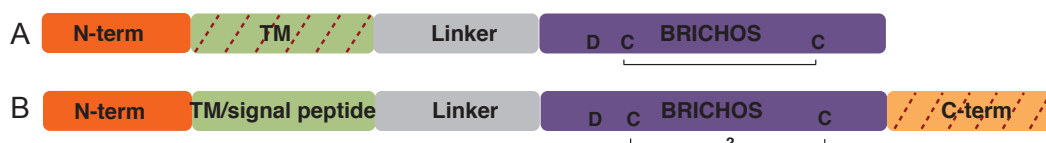


Figure 5. Schematic overview of BRICHOS structure. The N-terminal region is shown in dark orange, the TM region in green, the linker in grey, the BRICHOS domain in purple and the C-terminal region in beige. The aggregation prone regions in proSP-C (A) or other BRICHOS containing proteins (B) are marked with dashed lines.

Many proteins harbor segments with the possibility to form amyloid (Goldschmidt, et al., 2010), but it seems that evolution has found a way to prevent amyloid formation since only a small portion of these proteins form amyloid *in vivo*. Molecular chaperones, burial of aggregation prone segments, and intra-protein self-regulatory mechanisms have been proposed as endogenous ways to prevent amyloid disease in humans (Goldschmidt, et al., 2010, Landreh, et al., 2012). The BRICHOS domains have been proposed to bind the β -prone parts of their respective proprotein, thereby preventing aggregation (Johansson, et al., 2009a, Johansson, et al., 2009b, Peng, et al., 2010). This is supported by data showing that mutations in proSP-C BRICHOS, disabling BRICHOS function, lead to formation of amyloid of the aggregation prone TM region and ILD (Sipe, et al., 2014, H. Willander, et al., 2012a). Moreover, the BRICHOS domain has been shown to have anti-amyloid activity also against other peptides than its physiological clients (Nerelius, et al., 2009, Peng, et al., 2010). Indeed, both the proSP-C and Bri2 BRICHOS domains have been shown to reduce A β aggregation into fibrils *in vitro* (Arosio, et al., 2016, Cohen, et al., 2015, Peng, et al., 2010, H. Willander, et al., 2012). ProSP-C and Bri2 BRICHOS furthermore reduce A β aggregation and toxicity *in vivo* in a *Drosophila* model of A β amyloidosis (Hermansson, et al., 2014, Poska, et al., 2016). Moreover, unlike proSP-C BRICHOS, the BRICHOS domains from Bri2 and Bri3 are expressed in the CNS and could therefore represent attractive targets for up-regulation as A β anti-aggregation treatment in AD.

The only determined BRICHOS structure is the crystal structure of proSP-C BRICHOS, Figure 6. It is composed of five β -strands arranged in a mixed anti-parallel and parallel fashion, with two flanking α -helices. Molecular dynamic simulations suggest that one of the helices, α 1 can translocate and thereby expose the underlying face A of the β -sheet, which implicates face A as the binding site for possible substrates (H. Willander, et al., 2012a). Homology models of the human BRICHOS domains from each family showed that they are compatible with the proSP-C BRICHOS structure. Face A of the proSP-C BRICHOS contains mainly hydrophobic residues complementary to its hydrophobic target sequence, the TM region of SP-C. Bri2, and Bri3 BRICHOS instead have a charged face A and the proposed target sequences of Bri2 and Bri3, their C-terminal regions, are more charged indicating that this reflects the binding preferences of their BRICHOS domains (Knight, et al., 2013).

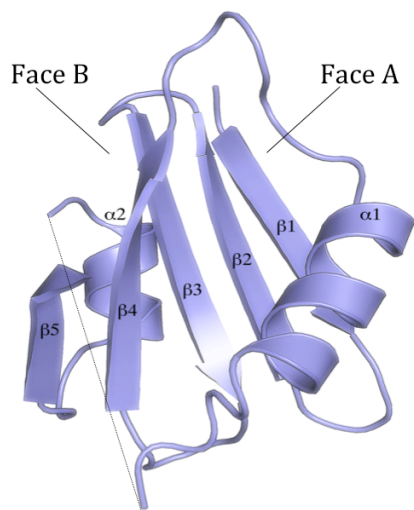


Figure 6. Crystal structure of proSP-C BRICHOS subunit. The structure shows a central five stranded β -sheet with two flanking α -helices. Face A and B is marked on opposite sides of the β -sheet. Adapted from Willander et al, 2012 (H. Willander, et al., 2012a).

1.8 PROSURFACTANT PROTEIN-C (PROSP-C)

ProSP-C is a proprotein expressed in the secretory pathway of alveolar type II cells, and contains four regions, an N-terminal region located in the cytosol, a TM region with high β -sheet propensity, a linker region and a BRICHOS domain facing the ER lumen (Mulugeta and Beers, 2006). Proteolytic cleavage of proSP-C generates the mature 35-residue SP-C, consisting of an α -helical poly-Val TM region, and an 8-residue N-terminal segment located in the cytosol (Johansson, et al., 1995, Johansson, et al., 1994). The SP-C peptide is secreted as part of the lung surfactant, into the alveolar space (Beers, et al., 1994, Whitsett and Weaver, 2002). SP-C is unique in the sense that although the primary translation product is a TM protein it is ultimately secreted as a lipophilic, mature peptide (Russo, et al., 1999).

1.8.1 ProSP-C BRICHOS in ILD

Mutations in the proSP-C gene lead to a recently discovered amyloid disease, ILD (Beers and Mulugeta, 2005, Nogee, et al., 2002, Nogee, et al., 2001, H. Willander, et al., 2012a). There are both inherited and spontaneous proSP-C mutations implicated in ILD (Hamvas, 2006) where some have been shown to give rise to amyloid deposits in the lung (Peca, et al., 2015, H. Willander, et al., 2012b). A majority of the ILD associated mutations are located in the linker region or in the BRICHOS domain, and several of these mutations have been shown to lead to amyloid formation of the SP-C peptide (H. Willander, et al., 2012a). The TM part of SP-C has a discordant α -helix and is composed of mainly valine residues that have high β -sheet propensity (Kallberg, et al., 2001). Data suggests that the BRICHOS domain of proSP-C promotes correct folding and insertion into the membrane of the α -helical TM part of SP-C, preventing the formation of amyloid and ILD (Johansson, et al., 2009a, Johansson, et al.,

2009b,H. Willander, et al., 2012a). Moreover, native SP-C isolated from lung surfactant aggregates into amyloid fibrils *in vitro* that can be visualized by electron microscopy (EM), but co-incubation with proSP-C BRICHOS abrogates the SP-C fibril formation (Nerelius, et al., 2008).

1.8.2 Quaternary structure of proSP-C BRICHOS

E. coli expression of proSP-C from residue 59-197, comprising the linker region and the BRICHOS domain, generates mainly a trimer in solution. The determined crystal structure is built up of a homotrimer, and analytical ultracentrifugation and size exclusion chromatography (SEC) indicate a trimeric structure (Casals, et al., 2008,H Willander, et al., 2012). The trimer interface is evolutionarily conserved to the same degree as the folded part of proSP-C BRICHOS domain, indicating that the trimer is functionally relevant, and it has been suggested to act as an inactive storage form by shielding the proposed binding surface composed of the β -sheet, face A (see Figure 6) (H. Willander, et al., 2012a). However, the quaternary structure of proSP-C BRICHOS *in vivo* remains to be established. It is notable that low immunoreactivity of proSP-C BRICHOS was detected in lung homogenates from rat and lysates of cultured rat alveolar type II cells, and no immunoreactivity in rat lung surfactant has been found (Beers, et al., 1994,Beers and Lomax, 1995), suggesting that it is not secreted and that its steady-state concentrations are low.

1.8.3 ProSP-C BRICHOS and A β aggregation

SP-C is one of the most hydrophobic proteins known (Beers and Lomax, 1995), and the involvement of proSP-C BRICHOS in its folding led to the idea that the BRICHOS domain has anti-amyloid properties that can be used against other amyloidogenic peptides (Nerelius, et al., 2009,Willander, et al., 2011). It has since been shown that proSP-C BRICHOS interacts with A β 40 *in vitro*, keeping it in a monomeric unstructured state over an extended time period (2 weeks) (H Willander, et al., 2012) as well as reduces A β 40 and A β 42 aggregation into fibrils (Arosio, et al., 2016,Cohen, et al., 2015,Nerelius, et al., 2009,H Willander, et al., 2012). Moreover, data from surface plasmon resonance (SPR) measurements and immuno-electron microscopy (EM) analysis suggest that proSP-C BRICHOS binds to fibrillar, but not monomeric species of A β 42 (Cohen, et al., 2015). Using a *Drosophila* model of A β 42 aggregation, it was shown that proSP-C BRICHOS reduces A β aggregation *in vivo*, and more importantly it was shown that co-expression of BRICHOS abrogated the toxic effects of A β 42, as shown by improvements in locomotor activity and longevity (Hermansson, et al., 2014). A β 42 aggregation kinetics and electrophysiology experiments on mouse brain tissue, have shown that proSP-C BRICHOS reduces the generation of toxic A β oligomers by inhibiting the secondary nucleation step (Cohen, et al.,

2015, Kurudenkandy, et al., 2014), which likely underlies the positive effects observed *in vivo* (Hermansson, et al., 2014), see Figure 7.

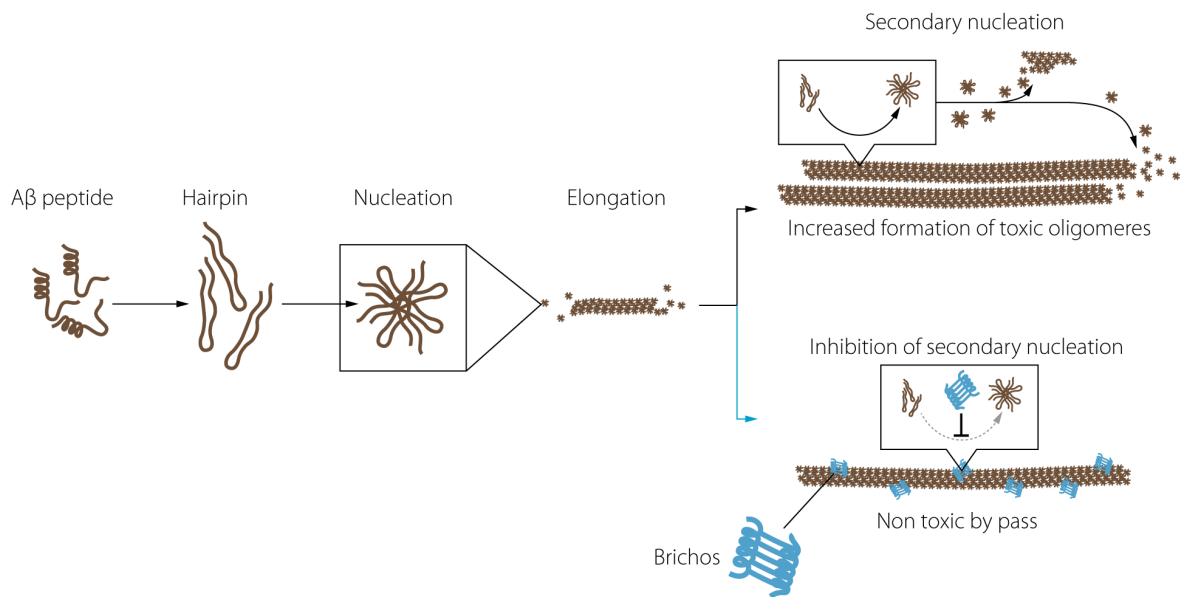


Figure 7. BRICHOS inhibiting secondary nucleation. The mechanism by which proSP-C BRICHOS inhibits Aβ aggregation is described by depicting Aβ aggregation in brown, and BRICHOS blocking the secondary nucleation pathway in blue. Used with permission, this figure was originally published in Ankarcrona et al, 2016 (Ankarcrona, 2016).

1.9 INTEGRAL MEMBRANE PROTEIN 2B (ITM2B), BRI2

The Bri2 protein is a 266 amino acid long, type II TM proprotein consisting of an N-terminal cytosolic part (residues 1-54), a TM region (residues 55-75), a linker (residues 76-136), a BRICHOS domain (residues 137-231) and a C-terminal region (residues 232-266). These delimitations are based on sequence alignments (Sanchez-Pulido, et al., 2002). However the sequence corresponding to the determined proSP-C BRICHOS crystal structure (H. Willander, et al., 2012a) aligned with the Bri2 and Bri3 sequences, suggest the Bri2 and Bri3 BRICHOS domains corresponds to residues ~130-231. Bri2 has an N-glycosylation site at asparagine 170 (Tsachaki, et al., 2011), and is expressed ubiquitously at high levels in brain, heart, placenta and pancreas (Vidal, et al., 1999). Processing of Bri2 releases a 23-residue peptide referred to as Bri23 (corresponding to residues 244-266 of Bri2) from the C-terminal region. Mutations in Bri2 give rise to release of extended, 34-residue C-terminal peptides, ABri or ADan. ABri and ADan deposit in the CNS in two rare amyloid diseases, familial British dementia (FBD) and familial Danish dementia (FDD), respectively (Cantlon, et al., 2015b). After the discovery of the pathogenic FBD and FDD mutations, and Bri2 as the precursor to the ABri and ADan peptides (Vidal, et al., 1999, Vidal, et al., 2000), furin was identified as responsible for the proteolytic cleavage releasing the C-terminal peptides (Kim, et al., 1999). Other proprotein-like convertases (PPCs) than furin are capable of processing Bri2, releasing C-terminal peptides, although data point toward furin as the more effective protease (Kim, et al., 1999, Kim, et al., 2000). Moreover, the BRICHOS domain can be shed

by ADAM10 and released into the extracellular space (Martin, et al., 2009, Martin, et al., 2008). The remaining membrane associated N-terminal fragment (NTF) of Bri2, is cleaved by intramembranous proteolysis by signal peptide peptidase-like (SPPL) proteases, SPPL2a and SPPL2b. This cleavage releases a Bri2 intracellular domain (ICD), as well as a secreted so called C-domain (Martin, et al., 2009, Martin, et al., 2008), not to be confused with the C-terminal peptide released by furin. The 23 amino acids situated just C-terminally of the TM domain (residues 76-98) are implicated in the SPPL cleavage of Bri2, and data points toward this cleavage taking place after ADAM10 mediated shedding (Martin, et al., 2009). Moreover, experimental data suggest that ADAM10 cleavage is not sequence specific, but the enzyme rather recognizes cleavage sites at certain distances from the plasma membrane (Sisodia, 1992). These data together suggest that ADAM10 releases the BRICHOS domain by cleavage somewhere between residues 99-136 in the linker region of Bri2. See Figure 8, for an overview of Bri2 processing.

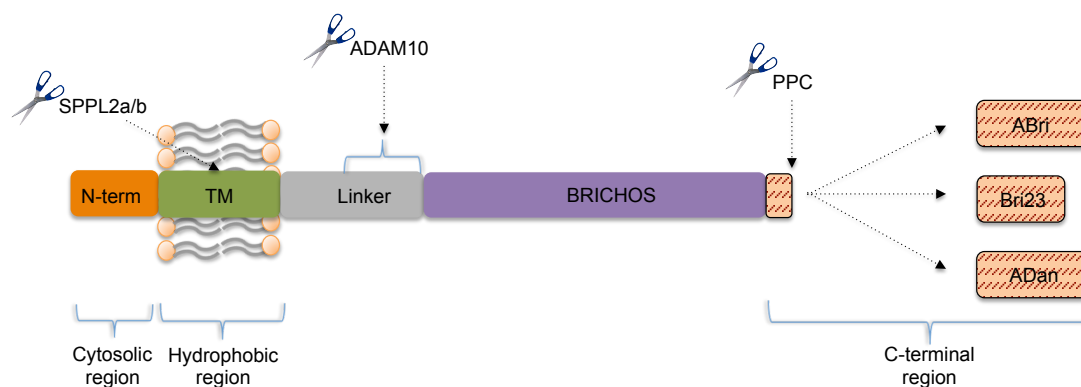


Figure 8. Schematic overview of Bri2 structure and processing. The N-terminal region is shown in orange, the TM region in green, the linker region in grey, the BRICHOS domain in purple and the C-terminal region in beige. Cleavage by SPPL2a/b in the TM region, by ADAM10 in the linker region and proprotein-like convertases (PPCs) in the C-terminal region is marked by scissor symbols.

1.9.1 Bri2 in familial British and Danish dementia, and links to A β PP

FBD is a rare disease that shares many similarities with AD, eventually leading to loss of memory and dementia (Mead, et al., 2000). Typical histological findings are amyloid deposition of ABri, cerebral amyloid angiopathy (CAA) and NFTs (Vidal, et al., 1999). FDD share similarities with FBD, but has different symptoms like development of cataracts and deafness, but eventually these patients also develop dementia. Histological findings include CAA, NFTs and hippocampal ADan amyloid plaques (Vidal, et al., 2000). In FBD the pathogenic mutation converts the stop codon in the gene for Bri2 to a codon for arginine, extending the open reading frame to include 11 additional amino acids residues, giving rise to the 34 residue long extended peptide, ABri. The FDD mutation leads to a 10-nucleotide duplication, causing a frame shift replacing the stop codon of Bri2, extending the peptide to another 34 residue long peptide. Both ABri and ADan are 11 residues longer than the non-pathogenic Bri2 C-terminal peptide, Bri23, but the additional residues share no sequence

similarity (Cantlon, et al., 2015b). A β 42 and A β 4-42 have been found in both fibrillar and non-fibrillar deposits of ADan in FDD (Tomidokoro, et al., 2005), and Bri2 has been found to deposit with A β plaques in AD (Del Campo, et al., 2014a). These observations suggest possible mechanistic links between the two diseases. It has been suggested that FBD and FDD are either caused by the aggregation of ABri and ADan respectively, and/or a loss-of function of mature Bri2 (Cantlon, et al., 2015b). Experimental support for both theories can be found. Data from FBD-Bri2 and FDD-Bri2 knock-in mice, as well as from human patients, show a reduction in Bri2 levels (Matsuda, et al., 2011, Tamayev, et al., 2010a), and knocking in WT Bri2 in FDD-Bri2 knock-in mice rescues negative effects on cognition (Tamayev, et al., 2010a, Tamayev, et al., 2010b). However, studies show that ABri and ADan aggregation *in vitro* causes cell toxicity (El-Agnaf, et al., 2001, Gibson, et al., 2004) and inhibits hippocampal LTP (Cantlon, et al., 2015a), similar to A β . The proposal that reduction in Bri2 levels causes FBD and FDD is based on the finding that Bri2 can affect A β PP processing.

It has been shown that Bri2 binds A β PP and modulates its processing leading to a reduction of secreted A β , both in *in vitro* (Fotinopoulou, et al., 2005, Matsuda, et al., 2005) and *in vivo* models (Matsuda, et al., 2008, Tamayev, et al., 2012). These studies imply that Bri2 restricts access of the secretases involved in A β PP cleavage, thereby regulating A β PP's processing. Other studies in cell cultures suggest that Bri2 interacts with BACE1, inducing its degradation through the lysosomal pathway, as well as reducing BACE1 mRNA levels through an unknown mechanism, thereby affecting A β PP processing by modulating the β -secretase levels (Tsachaki, et al., 2013). Moreover, some results indicate that residues 46-106 of Bri2 are responsible for binding the juxtamembrane and membrane spanning domains of A β PP (Fotinopoulou, et al., 2005), i.e the BRICHOS domain is not necessary for Bri2 interactions with A β PP in these experimental models (Fotinopoulou, et al., 2005, Matsuda, et al., 2005). However, an interesting observation is that the TM region of A β PP is involved in binding to Bri2 (Fotinopoulou, et al., 2005). The proSP-C BRICHOS domain together with its linker domain is implicated in correct folding and incorporation of the metastable α -helix of SP-C in the membrane (Johansson, et al., 2009a, Johansson, et al., 2009b). It is conceivable that the Bri2 linker together with its BRICHOS domain could have a role in incorporating the TM region of A β PP into the membrane.

1.9.2 Bri2 and A β aggregation

Mass spectrometry experiments show that Bri2 BRICHOS binds to A β 40 (Peng, et al., 2010), and reduces both A β 40 and A β 42 aggregation into fibrils *in vitro* (Arosio, et al., 2016, Peng, et al., 2010, H Willander, et al., 2012). A β aggregation kinetics show that Bri2 BRICHOS reduces A β aggregation by inhibiting both the secondary nucleation and elongation pathways (Arosio, et al., 2016), possibly making Bri2 BRICHOS a more efficient inhibitor of A β

aggregation, compared to proSP-C BRICHOS. A *D. melanogaster* model of A β 42 aggregation, indicates that Bri2 BRICHOS indeed is more efficient than proSP-C BRICHOS as inhibitor of A β 42 aggregation and toxicity *in vivo* (Poska, et al., 2016). Moreover, expressing full length Bri2 in A β PP transgenic mice reduced parenchymal A β 40 and A β 42 deposits as measured by biochemical and histochemical techniques. However, this data pointed towards the reduction of A β accumulation not being due to alterations in A β PP processing or altered A β production, and instead it was suggested that expression of Bri23 is required to achieve the effects on A β aggregation (Kim, et al., 2008). Another study expressing a Bri2 construct wherein the Bri23 peptide was swapped with A β 40/A β 42, resulted in high expression of A β 40 and A β 42. Interestingly these mice developed plaques much later than other A β PP mouse models with equivalent A β levels, and importantly, showed no cognitive deficits even after plaque formation (Kim, et al., 2013). Del Campo and coworkers proposed that Bri2 regulates the levels of IDE in the brain (Del Campo, et al., 2015), an enzyme responsible for degradation of A β (Farris, et al., 2003). It was hypothesized that Bri2 deposition in AD brain leads to a decrease in IDE, and thereby reduced degradation of A β (Del Campo, et al., 2015). All of these data together suggest that Bri2 affects the A β life cycle, but what region(s) of Bri2 that is/are responsible for the observed effects is not fully understood. Irrespective of what underlying mechanisms are involved, these studies collectively indicate a role of Bri2 in AD pathogenesis, which is worth further research.

1.10 INTEGRAL MEMBRANE PROTEIN 2C (ITM2C), BRI3

Bri3 is much less studied than Bri2 and it is, like Bri2, predicted to be a type II TM protein with an N-terminal region (residues 1-54), a TM region (residues 55-75), a linker region (residues 76-129), a BRICHOS domain (residues 130-230) and a C-terminal region (residues 231-267). Bri3 is only expressed in the CNS (Vidal, et al., 2001), and there is a predicted N-glycosylation site at residue 169. The sequence identity between Bri2 and Bri3 BRICHOS is ~60%, but experimental data support significant differences in Bri2 and Bri3 processing (Martin, et al., 2009, Martin, et al., 2008). Similar to Bri2, Bri3 can be processed by furin, releasing a C-terminal peptide corresponding to residues 243-267 (Wickham, et al., 2005). However, in a study investigating both Bri2 and Bri3 processing, no shedding of Bri3 BRICHOS by ADAM10 was detected (Martin, et al., 2009). As a result, no NTF was generated and no intramembranous proteolysis releasing an ICD of Bri3 could be identified (Martin, et al., 2009).

1.10.1 Bri3 and A β PP

Similarly to Bri2, Bri3 has been implicated in A β PP processing. One study shows co-immunoprecipitation and co-localization between Bri3 and BACE1 in neurons (Wickham, et al., 2005). Another study suggests a role for Bri3 in A β PP processing through blocking the

secretase cleavages of A β PP, without affecting the secretase activity towards other investigated substrates. This study showed that co-transfection of Bri3 and A β PP in a cell model leads to a reduction in A β secretion, and it was shown that A β PP could be co-immunoprecipitated with a Bri3 construct lacking the BRICHOS domain (Matsuda, et al., 2009). This indicates a similar binding to A β PP as suggested for Bri2 (Matsuda, et al., 2005), where the linker region is necessary for interaction with A β PP.

2 AIMS OF THE THESIS

The main aim of this thesis was to investigate the BRICHOS domain in relation to amyloid aggregation and Alzheimer's disease.

More specific aims were to:

- Investigate proSP-C BRICHOS quaternary structure in mammalian cells and modulate proSP-C BRICHOS activity towards A β fibrillation (**Paper I**).
- Find out if proSP-C BRICHOS has general anti-amyloid properties in mammalian cells (**Paper II**).
- Investigate possible physiological links between Bri2 and Bri3 BRICHOS and endogenous A β and study Bri3 BRICHOS activity against A β 42 fibril formation (**Paper III**).
- Determine if proSP-C and Bri2 BRICHOS reduce A β aggregation and toxicity *in vivo* in an A β PP/PS1 mouse model (**Paper IV**).

3 METHODOLOGY

A detailed description of the materials and methods used in this thesis can be found in papers **I-IV**. This section will only explain the less commonly used methods in the different studies, as well as the ethical considerations.

3.1 PROXIMITY LIGATION ASSAY (PLA)

PLA was used together with confocal microscopy to detect protein-protein interactions in mammalian cells (Paper **I**, **II** and **III**) or animal tissue (Paper **III** and **IV**). PLA is a sensitive antibody-based technique (Soderberg, et al., 2006) mainly used to detect, and strongly amplify single protein-protein interactions. The method works by coupling antibodies to oligonucleotides, termed PLUS and MINUS that are complementary to one another. When these are in close proximity of each other they can be ligated with ligase to form a circle. Polymerase, nucleotides as well as fluorescently labeled oligonucleotides are then added to the sample and a repeated, amplified signal is produced by rolling-circle amplification. The hybridized fluorescent probes can then be visualized with confocal microscopy by excitation and emission of the fluorophores, generating a highly amplified signal from each polymerization reaction.

In Paper **I**, monoclonal antibodies toward the V5 epitope was directly conjugated to oligonucleotides. Two different oligonucleotides, complementary to one another, termed PLUS and MINUS were conjugated to two different populations of anti-V5 antibody. The cDNA coding for the proteins of interest had been designed and cloned to express a V5 tag in their C-terminal. Stable cell lines expressing the V5-tagged protein were fixed and permeabilized, and the PLUS conjugated anti-V5 antibody as well as the MINUS conjugated anti-V5 antibody were added to the sample. When the antibodies with the complementary PLUS and MINUS oligonucleotides were in close proximity, which means less than 28 nm, PLA signal was obtained, meaning that signal was only obtained when two or more proteins with a V5 tag were in close proximity of each other. In Paper **II**, **III** and **IV** the proteins of interest were detected using primary antibodies from different species, against two different proteins. Complementary PLUS and MINUS oligonucleotides were coupled to secondary antibodies directed to the primary antibody species, e.g. anti-rabbit PLUS and anti-mouse MINUS. Using this approach signal is obtained when the two proteins of interest are at a distance less than 40 nm of each other.

When using the second approach, controls testing for secondary antibody specificity are important. Negative controls are performed using one of the primary antibodies and both secondary complementary antibodies, e.g. anti-rabbit PLUS and anti-mouse MINUS, for each primary antibody. In this way the background can be subtracted from true interaction signals. The primary antibody binding is controlled by using one of the primary antibodies, for

example a rabbit antibody, and two complementary secondary antibodies targeted against the same species, e.g. anti-rabbit PLUS and anti-rabbit MINUS. Signal will then be obtained for every single protein the primary antibody has bound. This can also be used for sensitive detection of protein subcellular location.

3.2 RAAV VECTOR INJECTION IN APP/PS1 TRANSGENIC MICE

The transgenic mouse model utilized in Paper **IV** is based on somatic brain transgenesis technology. Recombinant adeno-associated virus (rAAV) vectors are cloned, for expression of certain transgenes, and are then injected into cerebral ventricles of newborn mice. This generates widespread and permanent expression of the injected transgene. The rAAV expression method was developed with the aim of shortening the time span for generating specific transgenic mouse lines, as well as to reduce costs (Chakrabarty, et al., 2011, Kim, et al., 2008). The method in itself has been shown not to cause cognitive deficits, or other detrimental effects on mice development (Janus and Golde, 2014).

For the study in Paper **IV**, newborn mice transgenic for A β PP_{swe}/PS1 Δ E9 (A β PP/PS1) were injected with rAAV8 vectors encoding the signal peptide (SP)-proSP-C BRICHOS (residues 59-197) or SP-Bri2 BRICHOS (residues 90-236) domains. A negative control group of A β PP/PS1 mice were injected with rAAV8 vectors encoding green fluorescent protein (GFP), n=8 for each group. Quantitative polymerase chain reaction (PCR) was used to determine the genomic titer of each virus vector. Except for western blotting (WB) to verify BRICHOS overexpression, all biochemical analyses and cognitive tests were performed with male mice, 8 months of age, n=8 per group. The BRICHOS overexpression validation by WB was performed with male mice, 6-7 months of age, n=8, only transgenic for either proSP-C or Bri2 BRICHOS, and not A β PP/PS1.

3.3 ETHICAL CONSIDERATIONS

Human post-mortem brain tissue used in Paper **III**, was obtained from the Brain Bank at Karolinska Institutet, Sweden or Netherlands Brain Bank (VU medical Center), according to ethical permit 2011/962-31/1 and reference 2009/148 respectively. All animal procedures in Paper **III** were conducted in accordance with the regional ethics committee for animal research in Stockholm south, ethical permits S53-14, S151-10 and S75-13. Brain tissue from A β PP knock-out mice were kindly provided by Dr Hui Zheng, Baylor College of Medicine, U.S. All animal procedures in Paper **IV** were conducted in accordance with the Mayo Clinic Institutional Animal Care and Use Committee (IACUC), reference A40113, U.S.

4 RESULTS AND DISCUSSION

4.1 PROSP-C BRICHOS QUARTERNARY STRUCTURE IN MAMMALIAN CELLS, AND ACTIVITY OF THE BRICHOS DOMAIN AGAINST *IN VITRO* A β AGGREGATION (PAPER I, III)

4.1.1 ProSP-C BRICHOS quaternary structure in mammalian cells

The linker region and the BRICHOS domain of proSP-C, residues 59-197, is here referred to as proSP-C BRICHOS unless stated otherwise. The crystal structure of proSP-C BRICHOS was obtained after proteolytic cleavage with trypsin, revealing a homotrimeric structure. It was hypothesized that trimerization is a capping mechanism for protecting the hydrophobic face A in the monomer from exposing its proposed binding site (H. Willander, et al., 2012a), (see section 1.8.2).

In Paper I, stable HEK293 cell lines transfected for expression of a SP-proSP-C-V5 tag, or SP-proSP-C BRICHOS-V5 tag, were analyzed by PLA with directly oligonucleotide conjugated anti-V5 antibodies (see section 3.1), to determine the quaternary structure of proSP-C BRICHOS. The method was designed to distinguish between intracellular oligomers and monomers of the two different constructs, both containing a BRICHOS domain. The hypothesis was that proSP-C BRICHOS would form trimers more readily than the control, full-length (FL) proSP-C. The linker region of proSP-C together with its BRICHOS domain is involved in the folding and insertion of the TM region of proSP-C into the membrane (Johansson, et al., 2009a, Johansson, et al., 2009b, H. Willander, et al., 2012a). For the BRICHOS trimer to form under physiological conditions, proSP-C would likely have to be cleaved after insertion of the TM region, releasing the BRICHOS domain. Expressing a proSP-C BRICHOS construct where the TM part is not present it seemed plausible that the trimer would form directly after translation. However the result from PLA of the stable cell lines expressing proSP-C BRICHOS indicate that a trimer is not the dominant species in the secretory pathway, but a monomer. Moreover, no differences between the FL proSP-C or proSP-C BRICHOS constructs were detected. Control experiments detecting intracellular signals for both constructs ruled out insufficient expression as the reason for the lack of detectible trimers.

It is possible that although purified proSP-C BRICHOS expressed in *E. coli* readily forms a trimer in solution, this does not happen in a eukaryotic environment. However, other explanations are conceivable, such as proSP-C is not normally expressed in HEK293 cells, and it is possible that the same experiments in alveolar type II cells would have lead to a different result. Another explanation could be that the C-terminal V5-tag interfered with trimer formation, although the constructs were designed with this in mind, and the C-terminal is furthest away from the subunit interface (see (H. Willander, et al., 2012a)). The BRICHOS

domain of proSP-C could be degraded at a high rate so that trimer formation does not occur under physiological conditions, which is supported by data from alveolar type II cell lysates and rat lung homogenates showing low immunoreactivity for proSP-C BRICHOS (Beers, et al., 1994, Beers and Lomax, 1995). Lastly, an explanation for the discrepancy between the results from expression in eukaryotic cells and *E. coli* could be that 2 M urea is used for extraction during purification of *E. coli* produced proSP-C BRICHOS. It is possible that BRICHOS is monomeric also inside *E. coli* cells, and does not form trimers until cellular components are removed. It has been shown that proSP-C BRICHOS associates to phospholipid vesicles (Saenz, et al., 2015), and that amphipathic agents can dissociate the trimer into a monomer (Paper I, see below), supporting that the monomer could bind to biological membranes, for example in the secretory pathway.

4.1.2 Activity of *E. coli* expressed BRICHOS

Trypsin cleavage of proSP-C BRICHOS removes an unstructured region situated N-terminally of the BRICHOS domain. Except for a cleavage between helix 1 and 2, the proSP-C's BRICHOS domain is resistant against trypsin cleavage (Beers, et al., 1994, Beers and Lomax, 1995, Casals, et al., 2008). Limited proteolysis by trypsin increased the capacity of proSP-C BRICHOS to delay A β 42 aggregation, as measured by ThT fluorescence (Paper I). No increase in the monomer/trimer ratio after trypsination could be observed. The trypsination, cleaving between helix 1 and 2, hence did not lead to increased activity due to an increase in monomers but more likely to a more accessible binding site, similar to the unwinding of the helix 1 observed in molecular dynamics simulations (H. Willander, et al., 2012b), exposing face A (see Figure 6). 1,1'-bis (4-Anilino-5,5'-naphthalenesulfonate) (bis-ANS) is an amphipathic fluorescent probe that binds to proSP-C BRICHOS, in close proximity to the tyrosine residues (Casals, et al., 2008), that are predominately located in face A (see Figure 9). Bis-ANS increased the monomer/trimer ratio of proSP-C BRICHOS, as well as its ability to delay A β 42 fibril formation (Paper I). Replacing a small serine with a large and charged arginine in the trimer interface of BRICHOS also increased the monomer/trimer ratio of proSP-C BRICHOS, and its anti-amyloid activity (see Figure 9). These findings collectively support that the monomer is the active form. Chemical crosslinking of the trimer, caused a decrease of BRICHOS activity and its ability to bind to A β 42 fibrils, further supporting that the trimer is an inactive form. In contrast, both the WT proSP-C BRICHOS as well as the serine to arginine mutated form bind A β 42 fibrils as observed by immuno-transmission electron microscopy (immuno-TEM) (Paper I).

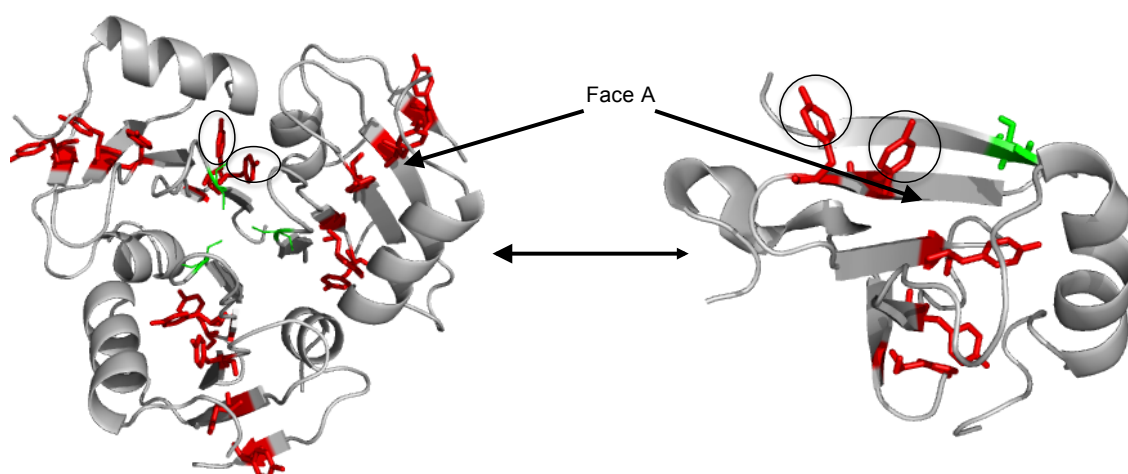


Figure 9. Structure of BRICHOS trimer (left) (PDB ID: 2yad) and monomer (right). The tyrosines in face A are highlighted in red, and the serine at position 95 is highlighted in green. The tyrosines that become exposed after dissociation from the trimer into monomer are circled. Used with permission, this figure was originally published in Biverstål et al, 2015 (Biverstal, et al., 2015).

Other BRICHOS domains like Bri2 (Poska, et al., 2016), and Bri3 BRICHOS (data not shown) also form oligomers and reduce A β 42 fibril formation *in vitro* (Paper III), and it is possible that small molecule compounds could enhance their activity by shifting the monomer/oligomer ratio. This is especially interesting for the BRICHOS domains that are expressed in the CNS, which therefore could be attractive targets for up-regulation in the search for treatment strategies of AD. It is difficult to get large molecules like proteins to cross the BBB and activation of endogenous proteins could therefore be an option.

4.2 INTRACELLULAR INTERACTIONS OF THE BRICHOS DOMAIN WITH AMYLOID PROTEINS/PEPTIDES (PAPER II, III AND IV)

4.2.1 ProSP-C BRICHOS general anti-amyloid properties

In Paper II, the effect of a BRICHOS domain on artificial β -sheet proteins (β 17, β 23) designed to form amyloid-like fibrils (West, et al., 1999) were studied. These designed β -sheet proteins have earlier been used to study cytotoxicity associated with amyloid-like aggregation in the cytosol of a human cell line and were then shown to cause cytotoxicity according to their predicted β -sheet propensity, β 23> β 17 (Olzscha, et al., 2011). Co-expression of proSP-C BRICHOS and proSP-C (residues 1-58) *in trans*, in the secretory pathway of HEK293 cells, stabilizes the otherwise aggregation prone proSP-C (1-58) from degradation (Johansson, et al., 2009a). It has been proposed that a β -hairpin structure, an early motif in amyloid aggregation, is a target motif for BRICHOS domains (Knight, et al., 2013). The model β -proteins most likely form this motif, and we therefore redesigned them

for co-expression *in trans* with the proSP-C BRICHOS domain in the secretory pathway of HEK293 cells, in order to study the anti-amyloid activity of proSP-C BRICHOS.

Co-expression of proSP-C BRICHOS leads to an increase of soluble β -protein, without affecting α -helical control protein levels. BRICHOS could not be detected when expressed with β 23, and therefore focus was centered on β 17. β 17 accumulated in inclusion body-like structures and BRICHOS co-expression reduced their size. Co-localization of BRICHOS and β 17 in inclusion body-like structures were detected by immunofluorescence, and a complex could be detected between β 17 and BRICHOS with both immunoprecipitation and PLA. Proteasome involvement in β 17 degradation was investigated with MG132 (a proteasome inhibitor) treatment and WB, and it was found that β 17 was partly degraded by the proteasome, but co-expression of BRICHOS reduced this degradation pathway. Cells expressing β 17 without BRICHOS, contained ubiquitinated protein and formed aggresome-like structures, as detected by the ProteoStat assay. The ProteoStat dye (Shen, et al., 2011) cannot distinguish between inclusion-bodies formed in a microtubule-dependent or -independent manner, making it hard to distinguish between aggresomes or other types of protein aggregates, therefore non-transfected cells treated with proteasome inhibitor were used as control. The MG132 treated cells formed abundant structures that were stained with the ProteoStat dye, as well as with ubiquitin-antibodies. This control does not prove that the detected structures are aggresomes but suggest that the aggregated protein structures detected in Paper II, accumulate as an effect of a stressed cell and indicate proteasome involvement. Expression of β 17 alone gave rise to formation of aggresomes-like structures and ubiquitinated protein, suggesting that the ubiquitin-proteasomal system (UPS) is affected. Proteasome activity was further studied by analyzing the chymotrypsin-like activity in transfected cells and β 17 was found to decrease proteasome activity compared with non-transfected controls, whereas BRICHOS co-expression reduced the inhibitory effects of β 17 on proteasome activity.

The results in Paper II suggest that β 17 expression in the secretory pathway of HEK293 cells leads to the formation of inclusions in the ER, and to some β 17 being retrotranslocated to the cytosol where it is targeted for proteasome degradation by ubiquitination. The formation of ubiquitinated inclusions and structures stained with ProteoStat, together with inhibitory effects on proteasome activity suggest that the proteasome is having problems degrading β 17. With BRICHOS co-expression, a model is suggested where β 17's accumulation into inclusions is reduced along with reduced need for retrotranslocation to the cytosol, ubiquitination, and proteasome degradation (see Figure 10).

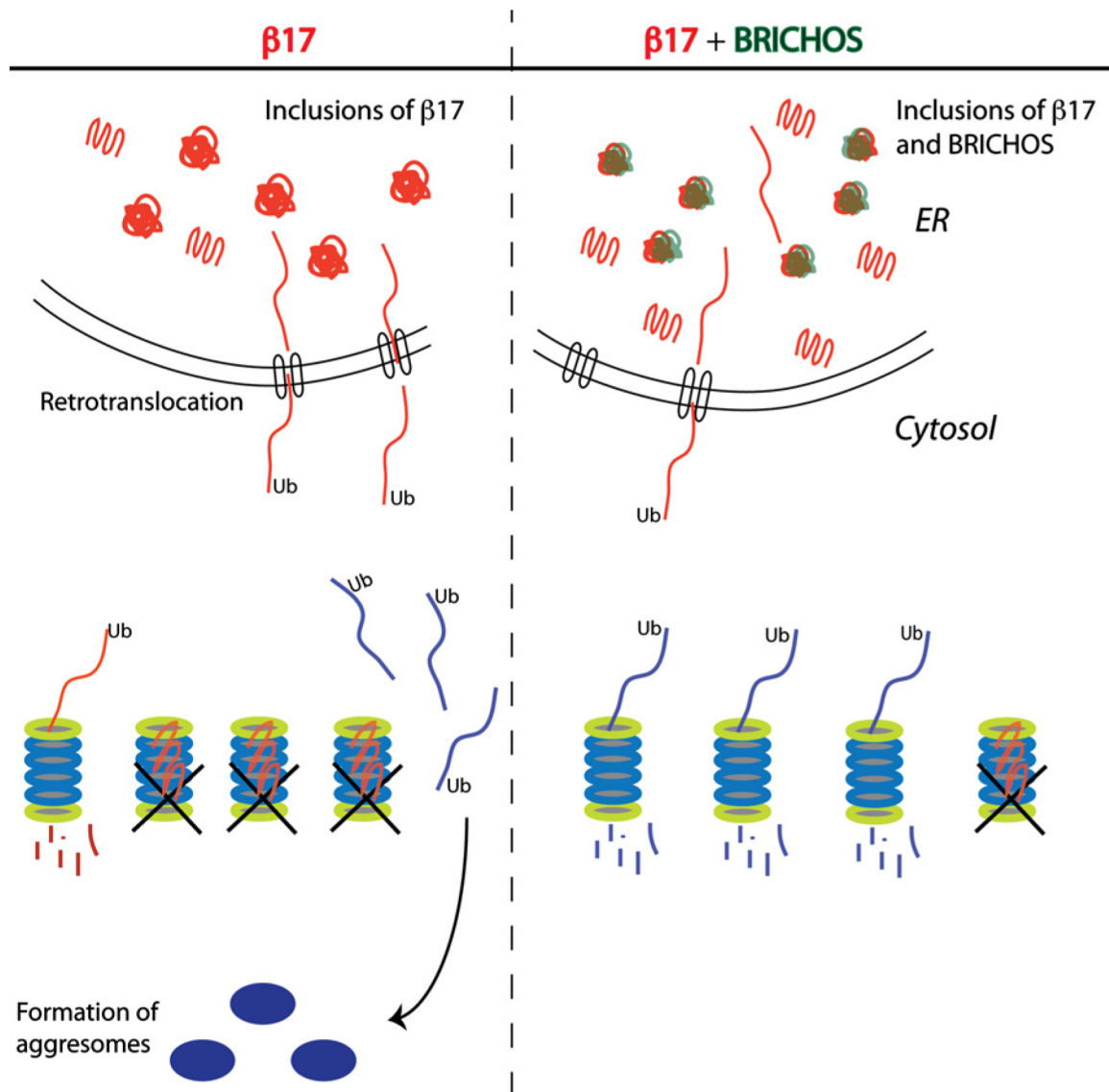


Figure 10. Model of $\beta 17$ and BRICHOS. Left: $\beta 17$ protein expressed in the secretory pathway aggregates and forms inclusions in the ER. $\beta 17$ is retrotranslocated to the cytosol and ubiquitinated (Ub) for degradation by the proteasome. Some $\beta 17$ is degraded but the proteasome is blocked with misfolded $\beta 17$, and its activity decreases. Proteasomal inhibition leads to accumulation of aggresomes, as detected using the Proteostat dye. Right: BRICHOS decreases the aggregation of $\beta 17$, resulting in less retrotranslocated $\beta 17$. BRICHOS binds to $\beta 17$, which leads to smaller inclusions that contain both BRICHOS and $\beta 17$. When less $\beta 17$ is targeted to the proteasome, the proteasomal inhibition is reduced and no aggresomes are formed. Used with permission, this figure was originally published in Dolfe et al, 2016 (Dolfe, et al., 2016).

4.2.2 Interaction between Bri2 and Bri3 BRICHOS with A β in neurons

In Paper III, Bri2 and Bri3 BRICHOS were studied in relation to A β and AD. Bri2 and Bri3 have been shown to bind A β PP and decrease A β secretion in transgenic cell lines, and therefore we decided to study endogenous Bri2 and Bri3 interactions with A β PP and A β in primary neurons from WT mice, using PLA (see section 3.1). Their interaction with A β PP could be verified, and interactions with A β 40 were also detected. Moreover Bri2 BRICHOS, without its linker region, hence after proteolytic release of the BRICHOS domain, interacted with endogenous A β 42 in WT primary neurons. Both isolated recombinant Bri2 and Bri3

BRICHOS domains, inhibit *in vitro* A β 42 fibril formation. Therefore we wanted to determine if Bri3 as well as Bri2 BRICHOS interacted specifically with A β in cells. It was found that Bri3 has abundant interactions with A β 42, however in contrast to Bri2, Bri3 BRICHOS was not found shed from neurons, and it could not be made certain if the BRICHOS domain or/and other parts of Bri3 are responsible for the interaction with A β . Considering that Bri3 BRICHOS inhibits A β 42 fibril formation, similar to Bri2 BRICHOS, it seems plausible that the BRICHOS domain is responsible for binding A β . Mouse brain tissue, transgenic for the arctic mutation, tgA β PParc (Ronnback, et al., 2012) were used to verify the interaction with A β PP and showed abundant signals in the CA1 region. A complementary control in A β PP knockout mice were performed and no interaction could be detected. Unspecific signal is a potential problem in this study and the results are dependent on the specificity of the antibodies. One factor that cannot be ruled out is that although A β and the BRICHOS domain are in close proximity to one another, <40 nm (see section 3.1), they could both bind a third protein and not actually each other. At least the control experiments, especially from the A β PP knockout mice show that the detected interaction is directly dependent on A β PP expression and not due to unspecific antibody binding. Another aspect, which is not investigated in Paper **III**, is whether Bri2 and Bri3 BRICHOS interact with monomeric or oligomeric A β . Previous data support that proSP-C BRICHOS bind fibrillar and not monomeric A β 42 (Cohen, et al., 2015). However these experiments have not been performed for Bri2 and Bri3 BRICHOS. Moreover, experiments show that Bri2 BRICHOS inhibits both elongation and secondary nucleation during A β fibril formation, whilst proSP-C BRICHOS inhibits only secondary nucleation (Arosio, et al., 2016), suggesting that Bri2/Bri3 BRICHOS potentially inhibit A β fibril formation by blocking different pathways than proSP-C BRICHOS. Studies in transgenic cells indicate that the binding of Bri2 and Bri3 to A β PP is mediated by the linker region and not the BRICHOS domain, these studies also suggest that the linker region is responsible for effects on A β PP processing (see section 1.9-1.10.1). It is therefore interesting to note that expressing a Bri2 construct from residue 90-236, including part of the linker region (residues ~76-129) and the BRICHOS domain (residues ~130-230) *in vivo* in a mouse model of A β PP/PS1 overexpression, had no effects on A β PP processing, whilst reducing A β aggregation and subsequent neuropathological effects (Paper **IV**). The results in Paper **III** and **IV** together support that the BRICHOS domain in itself, without the full linker region, is important for binding to A β , and reducing its aggregation.

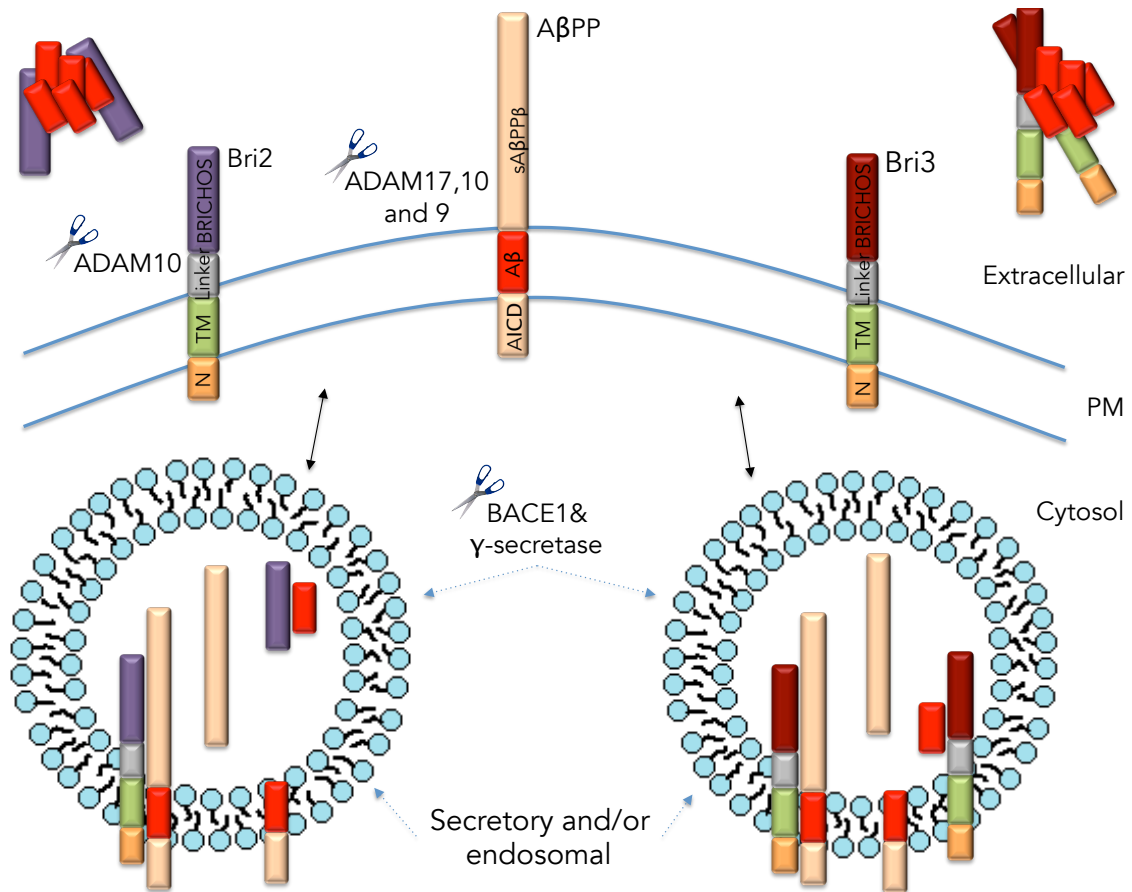


Figure 11. Model of A β PP and Bri2/Bri3 interactions. A β PP is produced in the ER and then transported through the trans-golgi network (TGN) to the plasma membrane (PM) and/or the endosomal pathway. At the plasma membrane A β PP is cleaved by α -secretase and/or internalized. A β PP is cleaved by BACE1 and γ -secretase mainly in the endosomal pathway, but also in the ER and TGN, hence, A β can be generated in both the secretory and endosomal pathway (van der Kant and Goldstein, 2015). Bri2 is generated in the ER and similarly to A β PP it could be transported through the TGN to the PM or endosomal pathway. At the PM it can be cleaved by ADAM10 and/or internalized. Bri2 still containing its linker region, interacts with A β PP (Paper III) and possibly other A β PP processing products, although this was not investigated in Paper III. Processed Bri2 BRICHOS interacts with A β (Paper III). Bri2 BRICHOS could come into contact with luminal A β , either in the TGN or the endosomal pathway. Extracellular A β and Bri2 BRICHOS can be found deposited in plaques (Paper IV). Bri3 is biosynthesized in the ER and similarly to A β PP and Bri2 it could be transported to the PM or the endosomal pathway. Bri3 BRICHOS is not shed (Paper III), but could become internalized. Bri3 interacts with A β PP (Paper III), and possibly with other processing products. Bri3 interacts with A β (Paper III) and similarly to Bri2 it could come into contact with luminal A β either in the TGN or in the endosomal pathway. Bri3 is found co-localized with plaques in AD brain (Paper III) and is possibly released with A β by exosomes. The A β PP processing products sA β PP α , C83 and p3, are left out of the illustration.

4.3 BRICHOS EFFECTS ON *IN VIVO* A β AGGREGATION (PAPER III AND IV)

Bri2 immunoreactivity increases in AD patients, and has been found deposited around plaques (Del Campo, et al., 2014b). Therefore Bri3 accumulation was investigated with immunohistochemistry (IHC) in Paper III, and was found deposited around plaques. Moreover, the levels of Bri3 are reduced in cortex and hippocampus of AD brains as measured by WB. A possible explanation could be that mainly the soluble levels are measured and could decline as insoluble Bri3 accumulates around/in plaques. This result is in contrast to Bri2 immunoreactivity, which increases with deposition around plaques, and it is

possible that the differences in processing of the Bri2 and Bri3 proteins are related to this discrepancy (see Figure 11). Bri2 BRICHOS is normally shed and could accumulate extracellularly in AD brains as a result of deposition around plaques, perhaps a sign of Bri2 BRICHOS trying to chaperone aggregating A β and up-regulation of Bri2. Bri3 on the other hand is seemingly not normally shed, and could deposit around plaques in AD brains as a result of release from dying neurons or in exosomes, decreasing the soluble Bri3 population. These speculations need to be addressed in future experiments.

In Paper IV, A β PP/PS1 transgenic mice were injected with either proSP-C, or Bri2 BRICHOS rAAV vectors to generate co-expressing mice for studying BRICHOS effects on *in vivo* A β aggregation. Controls were injected with a vector for GFP expression instead of the BRICHOS domain (see section 3.2). Measuring A β deposition by IHC and staining with Thioflavin-S, it was found that Bri2 BRICHOS significantly reduced amyloid deposition. ProSP-C BRICHOS also reduced amyloid deposition, although statistical significance could not be found. Enzyme-linked immunosorbent assay (ELISA) was used for further analysis of A β 40 and A β 42 levels. The samples were extracted to compare levels of different solubility, and there was a slight increase in phosphate buffered saline (PBS) soluble A β 40 and A β 42 with BRICHOS co-expression as compared to GFP controls. However, A β 40 and A β 42, soluble in RIPA buffer (containing detergents), significantly decreased. The RIPA-insoluble A β 40 and A β 42, solubilized by 5 M guanidine hydrochloride, decreased by ~30% or more with BRICHOS co-expression compared to GFP controls, see Figure 12. The PBS fraction is either intracellular or extracellular, not yet aggregated A β . The RIPA-insoluble fraction is likely mainly extracellular, aggregated A β , although this fraction could contain some aggregated intracellular A β . The RIPA-soluble fraction is likely at least partly membrane associated intracellular A β . Considering that the RIPA-soluble A β levels are decreased, it suggests that the effects of BRICHOS co-expression could start already intracellularly. No differences were found between the levels of analyzed A β PP processing products from the GFP control mice and the BRICHOS overexpressing mice, suggesting that the effects on A β levels and aggregation are due to other mechanisms than affecting A β PP processing.

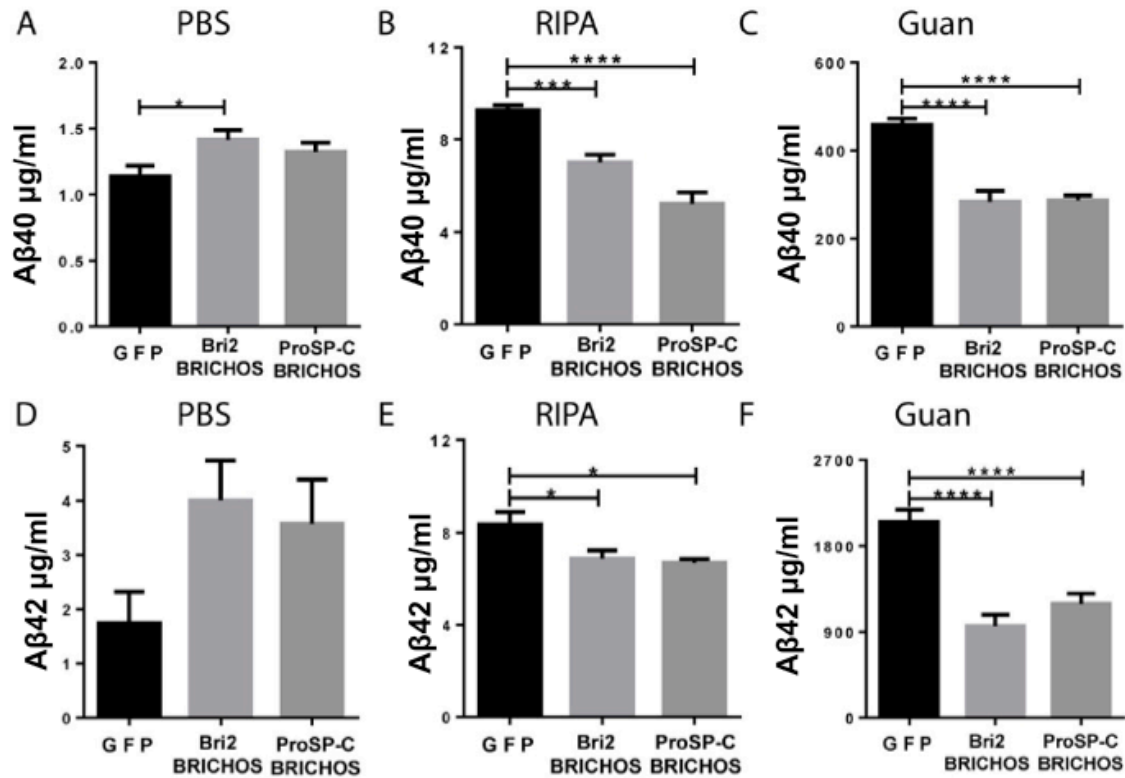


Figure 12. Aβ levels. Aβ40 levels in PBS (A), RIPA (B) or guanidine hydrochloride (Guan) (C). Aβ42 levels in PBS (D), RIPA (E) or guanidine hydrochloride (F).

When analyzing neuroinflammation by detection of the glial fibrillary acidic protein (GFAP) we found that both Bri2 and proSP-C BRICHOS overexpressing mice showed reduced neuroinflammation compared to GFP control mice. This is interesting considering that proSP-C BRICHOS did not have significant effects on the Aβ plaque load. ProSP-C BRICHOS has been found to only effect secondary nucleation, leading to the reduction of toxic oligomers (Cohen, et al., 2015). It is possible that although proSP-C BRICHOS did not significantly reduce Aβ plaque formation in 8-months old mice, it reduced the neuroinflammation occurring as a response to Aβ aggregation, perhaps as a result of reducing the formation of toxic oligomeric species. IHC showed that both Bri2 and proSP-C BRICHOS accumulate around Aβ plaques. Endogenous Bri2 is expressed in mouse brain and was not intentionally downregulated in this model, therefore controls using C-terminal and N-terminal antibodies were performed, to rule out other regions of Bri2 than BRICHOS accumulating around the plaques. Additional experiments verifying a direct interaction between the BRICHOS domains of proSP-C and Bri2 with Aβ42 in and around plaques were done by PLA in combination with Thioflavin-S staining. These experiments showed abundant interactions between Aβ42 and both proSP-C and Bri2 BRICHOS domains.

Lastly, BRICHOS effects on memory and learning compared to GFP overexpression in A β PP mice were analyzed, by testing mice behavior with the Morris-water maze. These experiments gave mixed results, where the first training sessions with a visible or hidden platform showed a trend but no significance for cognitive improvement with BRICHOS overexpression. But when entirely removing the platform and analyzing how much the mice had learned from previous training sessions, the time the mice spent in the area where the platform previously was situated, significantly increased suggesting improved retention of memories.

5 CONCLUSION AND FUTURE PERSPECTIVES

5.1 CONCLUSIONS

- ProSP-C BRICHOS is monomeric when expressed in HEK293 cells.
- Increasing the monomer/trimer ratio of proSP-C BRICHOS increases its activity towards inhibiting A β 42 fibril formation. Small molecule treatment could be a way to activate extracellularly added proSP-C BRICHOS and endogenous Bri2 and/or Bri3 BRICHOS.
- Bri3 BRICHOS effectively reduces *in vitro* A β 42 fibril formation similarly to proSP-C and Bri2 BRICHOS.
- ProSP-C BRICHOS reduces intracellular aggregation of designed amyloid-like proteins, thereby displays properties that can potentially be used against general amyloid aggregation.
- There are increased levels of Bri2 and reduced levels of Bri3 in AD brains. Both Bri2 and Bri3 deposit around plaques in AD brains, and there is a possible physiological link between intracellular A β with Bri2 and Bri3 BRICHOS in neurons.
- Bri2 and proSP-C BRICHOS reduces *in vivo* A β aggregation, neuroinflammation and negative effects on cognition, in a mouse model of A β amyloidosis.

5.2 FUTURE PERSPECTIVES

The work in this thesis support that the BRICHOS domain has properties that can be used against amyloid aggregation, *in vitro*, in cell models as well as *in vivo*. Whether this reflects an overall physiological role of the BRICHOS domain remains to be further investigated. Regardless of BRICHOS physiological role it could be interesting to try to use its properties as potential treatment of AD.

Expressing BRICHOS together with A β PP in mice shows that BRICHOS has potential for inhibiting *in vivo* A β aggregation and toxicity. However, it says little about how effective the BRICHOS domain could be if administered after disease onset, which is probably the ultimate goal when considering treatment of AD. It would be very interesting to see if it is possible to add recombinant BRICHOS into the circulatory system in mice, and have some BRICHOS cross the BBB. It will likely be a challenge to get the BRICHOS domain to cross the BBB, considering its size, but there are options to try. For example, a BRICHOS construct designed to bind receptors at the BBB, enabling transfer could be considered, as shown for the A β mAb158 antibody (Sehlin, et al., 2016). Another strategy could be to aim for activation of endogenous BRICHOS in the CNS, making it more effective in inhibiting A β aggregation. Similarly to proSP-C BRICHOS, Bri2 BRICHOS forms oligomeric states and recent unpublished data support that the low molecular weight oligomers and monomers are more effective in inhibiting A β fibril formation (Chen et al, to be published). It is conceivable that a small molecule treatment could be found that activates these Bri2 BRICHOS oligomers.

The results presented here suggest that BRICHOS interacts with both intracellular and extracellular A β , moreover that it has potential to inhibit both intracellular and extracellular amyloid aggregation. It is unclear what mechanisms are responsible for reducing A β levels, neuroinflammation and cognition in the mouse model, and where these effects are mainly taking place, intracellularly or extracellularly. It would be useful to study where BRICHOS is most effective, because this will affect what strategy in delivering BRICHOS that is of interest, out of a therapeutic viewpoint. Some of these questions would be answered by adding recombinant BRICHOS in mice with already developed amyloidosis, and study the effects. If this method decreases A β aggregation in the CNS and improve cognition then you could conclude that adding extracellular BRICHOS is sufficient to reduce *in vivo* A β aggregation and toxicity. An interesting experiment could be to repeat the study with the A β PP/PS1 mouse model, with FL Bri3 and determine if it is effective in reducing *in vivo* A β aggregation and subsequent effects on neuropathology, without shedding its BRICHOS domain into the extracellular space.

The interaction of Bri2 and Bri3 BRICHOS with A β , analyzed by PLA, could be of interest to detect by another method. Perhaps SPR experiments, immobilizing monomers, oligomers or fibrils of A β could determine what species of A β , that proSP-C, Bri2, and Bri3 BRICHOS preferably binds to. This could be useful in determining which of the BRICHOS domains to

focus on in relation to treatment of AD. Immuno-labeling of BRICHOS and A β with cryo-EM of neurons and/or synapses, or immunofluorescence and labeling with additional subcellular markers, using high-resolution microscopy could be useful in determining the location of BRICHOS interaction with A β in neurons.

To continue investigating whether there is a physiological role for Bri2 and Bri3 in A β PP, and A β 's lifecycle, is another subject worth studying. Is the deposition of Bri2 and Bri3 BRICHOS around plaques, and changed Bri2/Bri3 cortex levels in AD brains relevant to the development of the disease? It would be interesting to determine if BRICHOS levels are changed already at an early stage of the disease, with implications for its involvement in response to A β aggregation.

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